

# ***LABORATORY ASPECTS OF BIOLOGICAL WARFARE AGENTS***



*Edited  
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# Foreword

The three primary methods typical laboratories use for detection/diagnostics of infectious agents is:

- (1) cultivation of a viable agent,
- (2) use of the infected host to obtain evidence of infection (typically a serological response or other host response such as encephalitis), and
- (3) direct detection of the agent using laboratory methods such as amplification of the infectious agent's nucleic acids or an immunoassay targeted toward the infectious agent's proteins.

Direct cultivation of the agent is always the most discriminant evidence that an infectious agent is present. Direct cultivation, however, has several logistical and practical constraints. First is that not all agents are easily cultivated. Many of bacterial agents are fastidious, taking special media or conditions to support cultivation. Similarly, viral agents take special media and systems, and are not deployable outside a fixed facility. In addition, the time for culture takes anywhere from days to sometimes several weeks before results are available to enable a health care provider to take action based on the data.

The use of host responses, while sometimes the only evidence available, also has significant constraints on routine application, including the availability of the highly specific reagents necessary. Serological responses are rarely useful in direct patient treatment and only provide a look at what 'was.'

Direct detection, principally through nucleic acid amplification, has become the most significant method for patient treatment since the constraints to obtain meaningful results for patient treatment are the most minimal. While useful when a patient is displaying acute symptoms, direct detection is constrained by the availability of specific reagents for purported the disease.

All three systems have constrains, and acknowledgement of the constraints and mechanisms to understand and supplement the constraint with knowledge is the purpose of this manual.

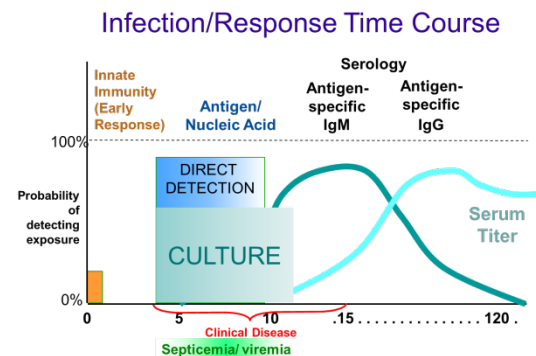


Figure 1. Cartoon of a typical infection-host response time course demonstrating potential diagnostic time points.

One of the best strategies to minimize the laboratorian from reporting a false-positive or a false-negative report is having sufficient information on which to base the final detection/diagnostic decision. Having multiple supportive answers improves the probability of the the final answer being correct. Obtaining multiple supportive answers is often referred to as orthogonal testing. Knowing the pros and cons of the individual assays helps understand the probability of obtaining a false-positive or a false-negative assay result. Orthogonal testing can not only be multiple assays, but also should include information from a physician's clinical assessment of the disease presentation or medical intelligence about infectious disease prevalence in the area of operations.



✓ Figure 2. Depiction of the concept of orthogonal testing. Each of the five pieces of information will help determine the correct answer with the intent to minimize the potential for false-negatives or false-positives.

May this manual provide you a reference to understand and provide good quality laboratory results for the protection of our war fighter's from biothreat agents, and infectious diseases in genera. Supporting the latter, information on the geographic dispersion of these agents is included in this manual to help provide a basis of information on the agent's occurrence naturally to help put in context any medical intelligence that is being developed.

# Bacillus anthracis

## OVERVIEW

*Bacillus anthracis*, the causative agent of anthrax, is a gram-positive, sporulating rod. The spores are the usual infective form. Naturally occurring anthrax is primarily a zoonotic disease of herbivores, with cattle, sheep, goats, and horses serving as the usual domesticated animal hosts, but other animals may be infected. Humans generally contract the disease when handling contaminated hair, wool, hides, flesh, blood, or excreta of infected animals and from manufactured products such as bone meal. Infection is introduced through scratches or abrasions of the skin, wounds, inhaling spores, eating insufficiently cooked infected meat, or by fly bites. Reports of suspected anthrax outbreaks date back to as early as 1250 BC. The study of anthrax and *B. anthracis* in the 1800s contributed greatly to our general understanding of infectious diseases. Much of Koch's postulates were derived from work on identifying the etiologic agent of anthrax and Louis Pasteur developed the first attenuated live vaccine for anthrax. The primary concern for intentional infection by this organism is through inhalation after aerosol dissemination of spores. All human populations are susceptible. The spores are very stable and may remain viable for many years in soil and water. They resist sunlight for varying periods.

*B. anthracis* is one of over 260+ different *Bacillus* species, but is readily distinguishable from most of the others by the production of a beta-hemolysin that is readily apparent on blood agar plates. *B. anthracis* contains 2 plasmids, pXO1 and pXO2 that impart virulence characteristics and serve as diagnostic markers for both immunoassay and nucleic acid assays. *B. anthracis* exists as both a vegetative cell and as an environmentally stable spore. Immunoassays will differ when testing for the vegetative cell or the spore. While immunoassay and nucleic acid analysis can be used for diagnostic confirmation, culture is required for confirmation.

## ORGANISM INFORMATION

### Taxonomy Information.

*B. anthracis* belongs to one of the most monomorphic species known. Isolates from whatever type of source or geographical location are almost identical phenotypically and genotypically. Phenotypically, strain differences are apparent in colonial morphology and LD<sub>50</sub> in animal tests.

*Bacillus*, as a genus, are an ubiquitous bacteria and hence are one of the best characterized genus. Even with the long history of classical microbiology, biochemistry, and more modern genomic and proteomic research approaches, the taxonomy of this genus continues to evolve.

There are over 260 species within the genus making distinction of the various species difficult. Typically *Bacillus* are considered more an environmental or industrial organism than a pathogen, hence little is done in a routine diagnostic laboratory.

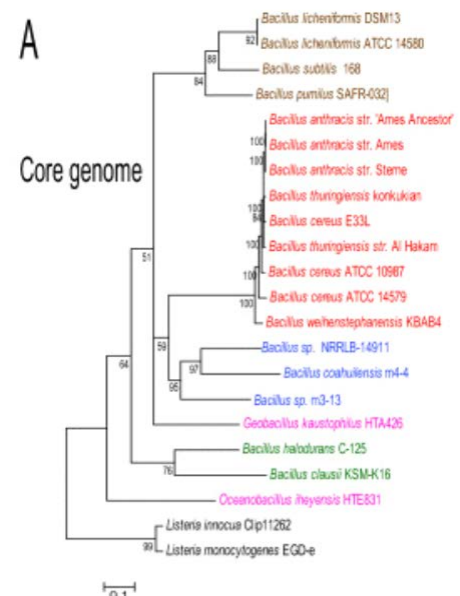
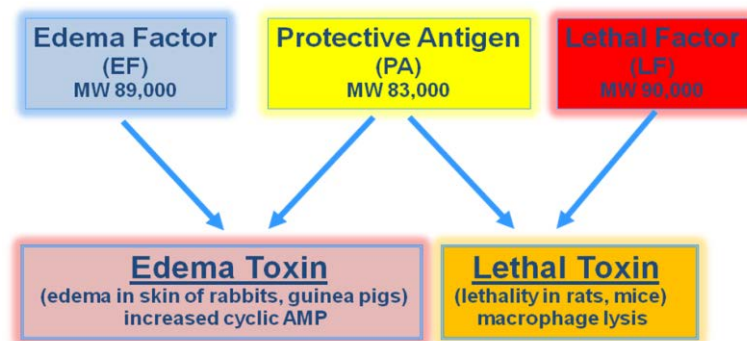


Figure 3. Phylogenetic constructs for *Bacillus* spp. (2).

## Virulence Factors.

*Bacillus anthracis* has two primary virulence factors; the polyglutamic acid capsule and the toxins (21). *B. anthracis* produces a relatively unique poly-D-glutamic acid capsule (unlike most other bacteria, which have polysaccharide capsules) (14, 36, 65) that has been implicated in virulence. The capsule is purported to inhibit host defense through inhibition of phagocytosis of the vegetative cells by macrophages. Many strains missing the 96 kb plasmid pXO2, which carries the capsule genes, but carry the pXO1 plasmid (182 kb) that harbors the anthrax toxin genes, exhibit reduced virulence in animal models. Non-encapsulated *B. anthracis* strains are readily phagocytosed by macrophages, whereas capsulated strains are not. Similar capsules are seen in *B. cereus*, *B. subtilis*, *B. licheniformis*, *B. megaterium*, *Sporosarcinia halophila*, and *Planococcus halophilus* and can cause confusion when capsule alone is used as a diagnostic marker.

The two toxins are composed of the interactions of three different proteins; edema factor (EF), lethal factor (LF) (8, 10) and protective antigen (PA).



Protective antigen (PA) is the central component of the three-part protein toxin complex and has a molecular mass of approximately 83,000 kD. After proteolytic activation and cleavage on the host cell surface, PA forms a membrane-inserting heptamer that passes the other toxic proteins (edema factor and lethal factor), into the cell.

Edema factor (EF) is an adenylate cyclase, an enzyme with key regulatory roles in essentially all cells. EF combines with protective antigen (PA) to form edema toxin. Edema toxin converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP); high intracellular levels of cAMP lead to impaired maintenance of water homeostasis and leads to the characteristic edema.

Lethal factor (LF) is a zinc metalloprotease (a protease that requires a metal). LF combines with PA to form lethal toxin. Lethal toxin apparently has three modes of action.

The first is thought to be the overproduction of cytokines (e.g., tumor necrosis factor and interleukin, which leads to lysis of macrophages. While the rapid release of the cytokines is what is thought to contribute to the suddenness of death with anthrax, in the mouse model it has not been shown to be the case (60). Lethal toxin also apparently impairs the function of dendritic cells by disrupting the mitogen-activated protein (MAP) kinase intracellular signaling network, which may suppress host immunity. The lethal toxin also has been shown to cause endothelial cell death, which may contribute to vascular destruction.

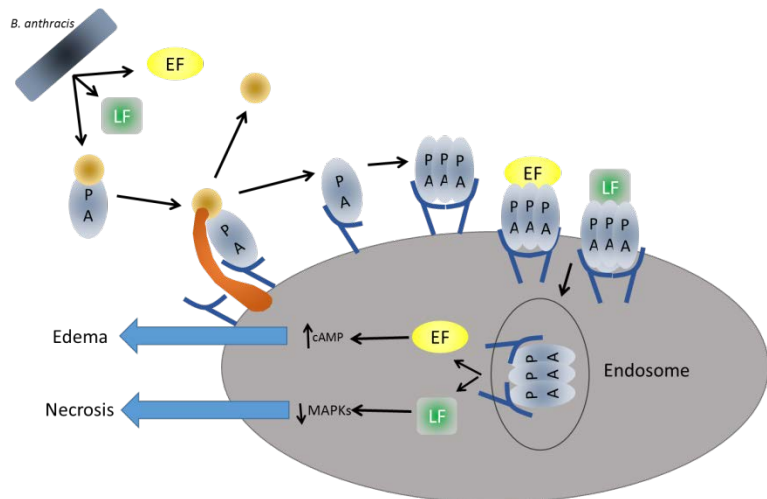


Figure 4. Cartoon of *B. anthracis* toxin's action on a cell.



### Epidemiology and Endemic Areas.

In most of the world, anthrax occurs sporadically (34, 56). In the United States, outbreaks in animals occur predominately in the Midwest (Kansas, Nebraska, North Dakota, South Dakota, Missouri, Texas and Oklahoma) and sporadically in California and Nevada. In the US, the microorganism remains endemic in the soil of Texas, Oklahoma, and the lower Mississippi valley.

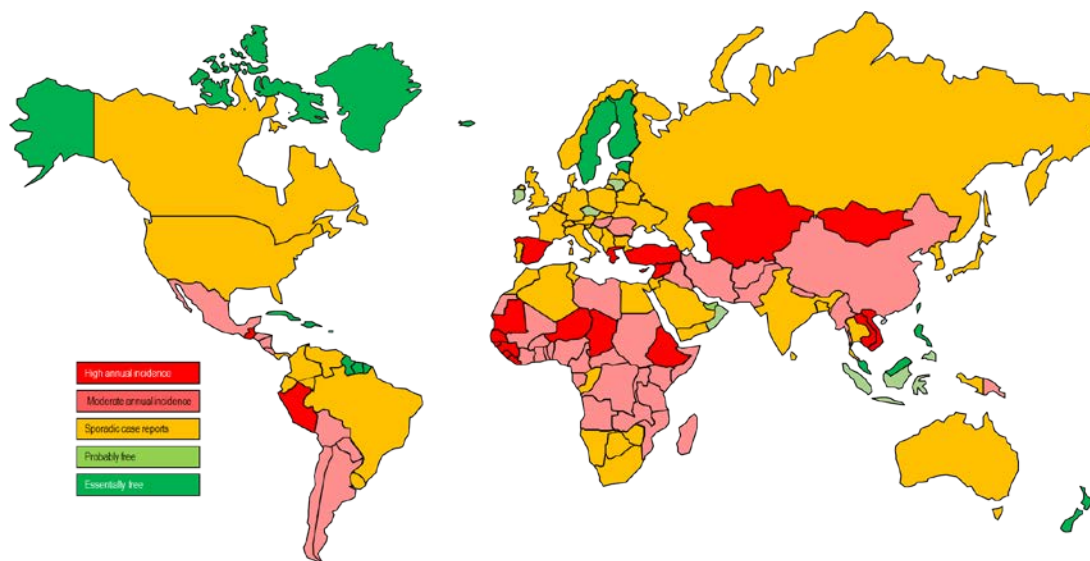


Figure 5. Global *B. anthracis* distribution.



Figure 6. Areas considered endemic for *B. anthracis* in the US.

## LABORATORY DIAGNOSTICS

### Biosafety Information.

Biosafety in Microbiological and Biomedical Laboratories (BMBL) Containment Recommendations (5th Edition): "BSL-2 practices, containment equipment, and facilities are recommended for activities using clinical materials and diagnostic quantities of infectious cultures. ABSL-2 practices, containment equipment and facilities are recommended for studies utilizing experimentally infected laboratory rodents. BSL-3 practices, containment equipment, and facilities are recommended for work involving production quantities or high concentrations of cultures, screening environmental samples (especially powders) from anthrax-contaminated locations, and for activities with a high potential for aerosol production."

### Diagnostic Information.

#### General.

*B. anthracis* is a gram-positive, encapsulated, endospore-forming, non-motile, rod-shaped bacterium, with a width of 1.0–1.2  $\mu\text{m}$  and a length of 3–5  $\mu\text{m}$ . It causes the disease named "anthrax", a common disease of livestock and occasionally humans.

#### Staining.

Direct examination of bacterial micromorphology will demonstrate broad encapsulated gram-positive rods. The Gram stain can be performed on appropriate clinical specimens (eg, vesicular fluid, swabs from cutaneous lesions, cerebrospinal fluid (CSF), pleural fluid, peritoneal fluid, sputum, or oropharyngeal ulcers). Endospores and free spores can be seen in preparations from cultures and generally are not seen in direct microscopic examination of patient samples.

Staining for bacterial capsules can be done with two different methods. The India ink stain is a classic microbiology method used to visualize bacterial capsules. Bacterial capsules will appear as well-defined clear zones around bacterial cells (see photo under confirmation section). Encapsulated *B. anthracis* typically display large capsules, but smaller capsules may be seen under conditions that do not favor capsule production. There is a polychrome methylene blue stain specific for capsule staining (M'Fadyean reaction). Using polychrome methylene blue the bacilli are stained blue and the capsule appears as red amorphous material around the chains of bacilli. In clinical samples such as blood, blood culture bottles, or cerebrospinal fluid, India ink stain can be performed directly.

Direct immunofluorescence assays (DFA) for cell-wall-associated polysaccharide and capsule produced by vegetative cells. Demonstration of both antigens can provide confirmatory identification (see photos under confirmation section). DFA used to be included in an official confirmatory testing protocol, but has recently been removed. Regardless, the technique is fast and accurate, with only minimal false positives with the cell-wall-associated polysaccharide and capsule, so far not in combination with each other.

There are two general methods for staining spores, malachite green and a modified Ziehl-Neelsen. The malachite green stain will show the spores, which are oval and non-bulging, stained green and the bacilli stained red. The modified Ziehl-Neelsen stain for spores will stain spores red and vegetative forms blue.

#### Metabolic Information:

*B. anthracis*, and many other *Bacillus* spp, grow well on most routine media within 18-24 h. Growth on sheep blood agar (SBA) and chocolate agar (CA) can be used for most specimens. *B. anthracis* does not grow on MacConkey agar (MAC). With occasional exceptions, it is generally easy to identify *B. anthracis* and to distinguish it from other *Bacillus* species. An isolate will have a characteristic colonial morphology on nutrient or blood agar, matt appearance, fairly flat, tacky, white or grey-white on blood agar, and often having curly tailing at the edges (a 'medusa head' appearance), and is non-hemolytic.

- (1) Optimal temperature: The optimal growth temperature for the organism is 35-37°C. Spores are produced readily on all ordinary laboratory media at 35-37°C.
- (2) Upper temperature: When grown above 45°C, the bacteria become attenuated or avirulent due to loss of the capsule genes.
- (3) Lower temperature: 12°C.
- (4) Optimal pH: Germination of spores outside an animal host may occur when the pH between 5 and 9. For vegetative cells, the optimal pH is between 7.0 and pH 7.4.

- (5) Note: Forms mucoid capsule when grown on agar with sodium bicarbonate and incubated in carbon dioxide-enriched atmosphere; capsule can be visualized with India ink preparation.
- (6) Note: Spores germinate when they fall into appropriate conditions; a temperature between about 8°C and 45°C, a pH between about 5 and 9, a relative humidity greater than 95% and the presence of adequate nutrients. Vegetative cells will only form spores after local nutrients are exhausted, such as when anthrax-infected body fluids are exposed to ambient air.

#### General Culturing Information

Nutrient agar (NA). After overnight incubation at 35-37°C, colonies are large 2–3 mm in diameter, irregular, raised, dull, opaque and grayish white with 'frosted glass' (ground glass) appearance. The edge of colonies may be curled or fringed edges with long interlacing chains of bacilli resembling curly locks. This is referred to as "medusa head appearance" but is not encountered as frequently as textbooks often suggest. The colony is membranous in consistency and hence not easily put into suspension.

Nutrient broth (NB). No turbidity or very fine floccular turbidity is seen with floccular deposit. The deposit comes up as silky strands on shaking the broth gently, because of the tendency to form long chains in vitro and tenacious growth character of the organism.

Heart infusion broth, amino acids, adenine and uracil, along with salts (HIBAAAUS): HIBAAAUS is an enhancement of heart infusion broth for the rapid germination of spores. The addition of amino acids, adenine, uracil, and salts provides improved induction of exosporulation and is a useful method of generating increased production of *B. anthracis* toxins. The formula for HIBAAAUS follows this chapter.

Sheep (horse) blood agar (SBA). Lack of hemolysis is the norm. Weak hemolysis may be observed very rarely under areas of confluent growth, which should not be confused with beta hemolysis. On sheep blood agar, the colonies are non-hemolytic, 2-3 mm in diameter, irregular, raised, opaque and grayish white with a 'frosted glass' appearance. The edge of the colonies is curled, or fringed, with the 'medusa head' appearance.

Leighton–Doi liquid medium, Schaeffer's Sporulation Medium (SSM) and New Sporulation Medium (NSM) are media developed specifically for the production of spores from *Bacillus* spp. . Leighton–Doi liquid medium is commonly used but usually is followed by Renografin (methyl glucamine) gradient centrifugation for spore clean-up (37). NSM, a solid medium, produces more spores for pXO1+ strains (with or without pXO2) whereas pXO2- strains and some strains within other species (ie. *B. cereus*, *B. thuringiensis*, *B. megaterium*, *B. mycoides*) do better in SSM. Usually parallel production in both medium is needed to determine which media produces the best sporulation for each strain.



Figure 7. *B. subtilis* (left) and *B. anthracis* (right); note zone of hemolysis around left culture; that is termed beta hemolysis.

### Selective Culturing Information

PLET Agar (Polymyxin B - Lysozyme - EDTA – Thallous acetate Agar). PLET Agar medium is the best selective medium for isolation and cultivation of *B. anthracis* from environmental specimens, animal products or clinical specimens. It is generally considered to inhibit other species of *Bacillus* and gram-negative organisms, but the specificity of PLET for *B. anthracis* is not complete. The beef heart infusion and tryptose are sources of carbon, nitrogen and other compounds necessary for growth. EDTA, thallous acetate and polymyxin are selective agents and inhibit most other microorganisms. Lysozyme attacks the cell wall of gram negative bacteria and inhibits them. Colonies of *B. anthracis* appear in 36-40 h after incubation at 35-37°C. The colonies are circular, creamy-white with a ground glass texture. Marston reported that 14 of 16 *B. anthracis* strains produced the expected morphology on PLET (88% sensitive) whereas 17 other *Bacillus* strains and 18 non-*Bacillus* spp. strains were used to evaluate the media's selectivity (ability to inhibit non-*B. anthracis* growth). PLET inhibited growth of 14/35 strains (40% selective) (46).

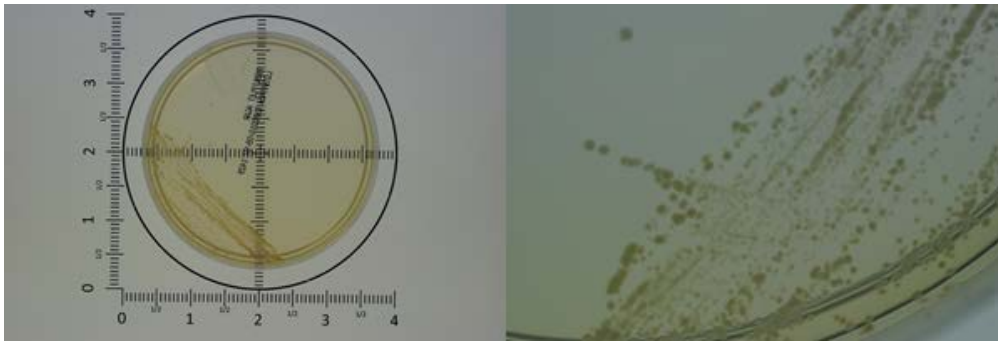


Figure 8. *B. anthracis* on PLET at 24 hrs.

Polymyxin egg yolk mannitol bromothymol blue agar (PEMBA), mannitol-egg yolk-polymyxin B [MEP (46) or MYP] are principally used for *B. cereus* (which is mannitol negative and hydrolyses lecithin) which produces characteristic blue colonies with a zone of precipitation. *B. anthracis* would produce a similar reaction but help rule out *Bacillus subtilis*.

Polymyxin blood agar. This medium is useful for testing old, decomposed or processed animal specimens or environmental specimens as it reduces or prevents growth of many gram-negative bacterial contaminants.

Bicarbonate agar. Principally used for enhancing capsule production for India ink or DFA procedures. Colonies are mucoid on this medium due to the capsule formation (65).

Anthraxis Chromogenic Agar (R & F Laboratory Products). *B. anthracis* colonies appear cream-colored or cream with a pale blue-teal center after 24 h incubation and blue-teal centers and a large white rim after 48 h incubation. The colonies should be 2-6 mm diameter, and have a rough, ground-glass texture and may or may not show "medusa-head" morphology. After 24 h and 48 h of incubation *B. cereus*/*B. thuringiensis* colonies will be a very dark teal-blue, with a thin cream rim, and 6-7 mm in diameter. After the 24 h incubation, colonies are marked and re-incubated for an additional 24 h. Any initially cream colored colony that has not developed a blue or teal center is not *B. anthracis*.

NOTE: It is common to work with direct specimens but heat-treated or alcohol-treated specimens can be used and reduces other non-*Bacillus* environmental contaminants.

*Bacillus* spp.

[illegible]

## Diagnostic Tests

Generally, *B. anthracis* is easily differentiated from other non-pathogenic *Bacillus* species, principally by the hemolysin reaction. Occasionally, other *Bacillus* species can cause confounding problems with individual tests. Assay deviations from the conventional diagnostic results for *B. anthracis* should be explored further to insure false-negatives are not reported (1).

Lecithinase, motility, and penicillin susceptibility are classically used to differentiate *Bacillus* species. While useful, lecithinase is not highly discriminatory, motility can be easily misinterpreted, and penicillin susceptibility does not take into account potentially genetically engineered or naturally occurring penicillin resistant strains, leading to potential mis-identification.

Gamma phage. Anthrax-specific phages were first isolated in the 1950s. The specifically-named “gamma phage” was first reported in 1955 and became the standard anthrax diagnostic phage; although it is clear that it is just one of a family of closely related anthrax phages. On a rare occasion, phage-negative *B. anthracis* or phage-positive *B. cereus* could be encountered. The phage must, therefore be used in conjunction with the other tests such as capsule production. A fresh preparation of *B. anthracis* is spread on the surface of sheep or horse blood agar plate with a sterile swab. A suspension of titrated phage is placed on the plate (typically in 2 quadrants) and the plate is incubated for 18 h at 35-37°C. Zones of clearing (may be partial with some satellite colonies present) are considered “sensitive to gamma phage” (54).

**Capsule.** Capsule formation can be demonstrated by transferring a pin-head quantity of growth from a suspect colony to approximately 2.5 ml of defibrinated blood or horse serum in a sterile test tube or small bottle and incubating 5 h to overnight. Theoretically, blood from any species should do but, in experience, there is



**Figure 9. Gamma phage testing for *B. anthracis*. The two zones of cell lysis indicate a positive/sensitive reaction to the gamma phage.**



great animal-to-animal variation and it is necessary to identify a source of reliable blood giving good capsule production. Defibrinated horse blood seems to be best. Alternatively, plate the suspect colony onto nutrient agar containing 0.7% sodium bicarbonate and incubate overnight at 37°C under CO<sub>2</sub> (a candle jar does very well for general purposes, but 20% CO<sub>2</sub> gas is typically used). Capsule-forming strains of *B. anthracis* form mucoid colonies (non-capsulating strains are rough). Smears of these can be stained with India ink or polychrome methylene blue stain (M'Fadyean reaction) and examined under a microscope.

Motility - *B. anthracis* is non-motile while most other *Bacillus* species are motile. This is typically done by microscopic observation of a 'hanging drop' slide.

Tenacity - *B. anthracis* colony has the consistency of beaten egg white when picked with an inoculating loop. Other *Bacillus* spp. colonies will collapse back on itself.

Penicillin Sensitivity - *B. anthracis* is sensitive to penicillin in vitro; most other species of *Bacillus* are resistant to penicillin. A fresh preparation of *B. anthracis* is spread on the surface of a Mueller Hinton agar/blood agar plate with a sterile swab. A penicillin G 10 unit disc is placed on the plate and the plate is incubated for 18 h at 35-37°C. The zone of inhibition around the disc should be around 29mm. NOTE: If there is any suspicion that *B. anthracis* genetic manipulation has occurred, penicillin sensitivity would be one of the first genetic modifications that could be induced.



Figure 10. India ink preparation for *B. anthracis* capsule (zone around cell).

Differential characteristics of <i>Bacillus anthracis</i> and other non-pathogenic <i>Bacillus</i> species		
Characteristic	<i>B. anthracis</i>	Non-pathogenic <i>Bacillus</i> species
<b>Capsule</b>	Present in clinical samples or laboratory induced	Absent <sup>2</sup>
<b>Lecithinase</b>	Positive (weak)	<i>B. cereus</i> , <i>B. mycoides</i> , <i>B. thuringiensis</i> positive, all others negative
<b>Motility</b>	Negative	Positive
<b>Penicillin susceptibility</b>	Sensitive	Resistant
<b>Hemolysis on blood agar plate</b>	Non-hemolytic	Hemolytic
<b>Gelatin liquefaction</b>	Slow	Rapid
<b>Susceptibility to penicillin</b>	Susceptible <sup>1</sup>	Generally resistant
<b>Susceptibility to gamma phage</b>	Susceptible (95%)	Resistant (95%)
<b>Pathogenicity</b>	Pathogenic	Generally non-pathogenic
<sup>1</sup> consideration for genetically manipulated strains that could be resistant		
<sup>2</sup> Certain other <i>Bacillus</i> species can produce a polypeptide capsule but these will not be typically found in clinical specimens and will not produce this capsule under normal laboratory culture conditions.		

Commercial Identification Systems (see Appendix E for technical descriptions of the methods of the methods)

Fatty acid methyl ester (FAME) analysis (see Appendix E. Commercial Identification Systems for a description): A dangerous pathogens database is available as a separate library that includes the data entries to identify the *B. anthracis* fatty acid profile. The determination of *B. anthracis* by this system has been approved as an AOAC Official Method (58). The system does a good job of resolving *B. anthracis* from the other *Bacillus* spp. but cannot differentiate between the highly homogeneous group *B. cereus* and *B. thuringiensis*, two commonly encountered near neighbors (3).

**Vitek:** The Vitek 2, using the *Bacillus* card (BCL; typically used in industrial but not clinical laboratories), will identify 42 taxa, representing six genera (*Aneurinibacillus*(1), *Bacillus*(18), *Brevibacillus*(8), *Geobacillus*(3), *Paenibacillus*(10) and *Virgibacillus*(2)). *B. anthracis* is included and identified separately from the other species in *B. cereus* group (*B. cereus*, *B. thuringiensis* and *B. mesenteroides*). bioMerieux Vitek claims a 100% identification of *B. anthracis* but that claim has not been independently substantiated. With internal work at USAMRIID, Vitek gave the correct answer for *B. anthracis* only about 15% of the time, with an incorrect *Bacillus firmus* result about 15% of the time. Other results for *B. anthracis* were low discrimination (13%), unidentified organism (18%), and the rest of the results being other results with less frequency (total of 945 tests run).

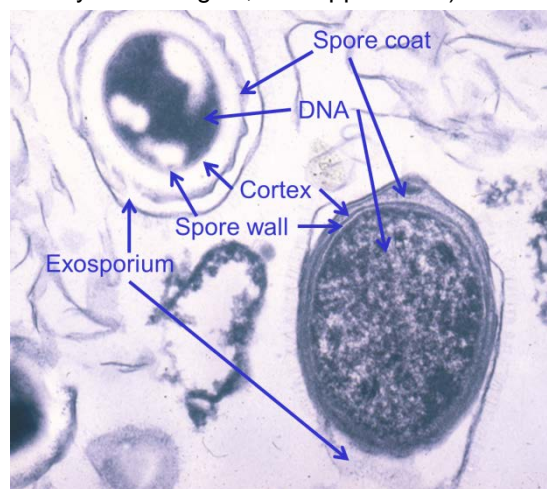
**Biolog:** The GN2 system had a Dangerous Pathogens database for identification of *B. anthracis*, and in general, it worked for the identification of *Bacillus* species, if care was taken using the 'dry tube' method. There were issues of close *Bacillus* being misidentified, so care had to be used in the interpretation of the identification and compared to other data (64). The new GEN III redox chemistry is applicable to both gram-negative and gram-positive bacteria and uses the ability of the cell to metabolize classes of biochemical and other physiological properties such as pH, salt and lactic acid tolerance, reducing power, and chemical sensitivity. While the GN2 system had a Dangerous Pathogens database for identification of *B. anthracis*, the current GENIII does not. Even with the new substrates, GENIII cannot separate the identifications of *B. cereus* and *B. thuringiensis* and would probably have some difficulties with absolute *B. anthracis* identifications.

### Immunoassay Tests.

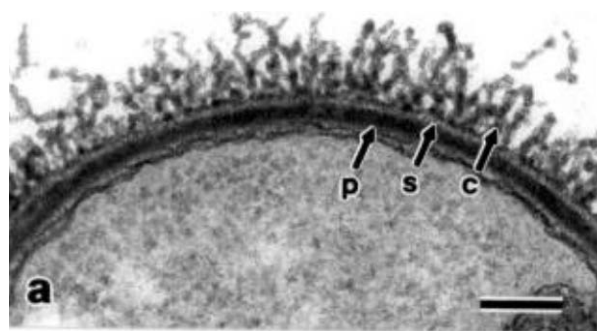
**NOTE:** For a short explanation of the principles of the assay technologies, see Appendix C).

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology). In addition, for *B. anthracis*, direct detection of the organism can be either detection of the vegetative cell or the spore phase. Immunoassays for *B. anthracis* vegetative cells or the spores will be inherently different since the exterior surface of each is completely different. In the vegetative form, the capsule (N-acetylglucosamine-galactose polysaccharide), edema factor (EF), lethal factor (LF) or protective antigen (PA) are all are good, and largely specific, immunoassay targets. Many monoclonal and polyclonal antibodies have been produced to these antigens and most perform with limited problems. In the spore form, specific proteins for *B. anthracis* are both harder to identify and produce. Often extractable antigens 1 (EA1) and 2 (EA2), with molecular masses of 91 and 62 kd, are used for spore detection. These proteins, however, are also present in other *Bacillus* spp. and are not related to the *Bacillus* spore, potentially causing false-positive reactions when assaying for *B. anthracis* spores (16, 39, 40).

In the *B. anthracis* vegetative cell, several physical structures can interfere with specific assays. The capsule can cause a physical barrier to surface layers of protein. Beneath the capsule is a proteinaceous 'S-layer', composed of two glycoprotein subunits (extractable antigen 1 (EA1) and surface array protein (SAP)) that cover the bacterial cell surface. It has been demonstrated that the S-layer can physically mask the negatively charged peptidoglycan. Beneath the S-layer is the peptidoglycan layer, comprising the cell wall. EA1 protein appears to be coded by chromosomal DNA, whereas the EA2 protein was only detected in strains that possessed the pXO1 toxin plasmid (11). Anti-EA1 reacted strongly with non-encapsulated vegetative cells of most *B. anthracis* and with some strains of *B. cereus* and *B. thuringiensis*.



**Figure 11. Electron micrograph of *B. anthracis* spores with chief components labelled.**



**Figure 12. Electron micrograph of *B. anthracis* vegetative cell layers showing the peptidoglycan layer (p), S-layer (s), and capsule (c).**

The BclA (*Bacillus*-collagen-like protein of *B. anthracis*) gene codes for the protein backbone of the exosporium glycoprotein. It has been noted that the BclA gene is also present in the genome of *B. cereus* and *B. thuringiensis*, but the size differences among the BclA proteins, both for species and strains (due to different numbers of amino acid repeats) might be a good target for immunoassay development, but the specificity of many BclA assays has not been fully reported. BclA proteins are one of the immunodominant sites on the surface of *B. anthracis* spores (66).

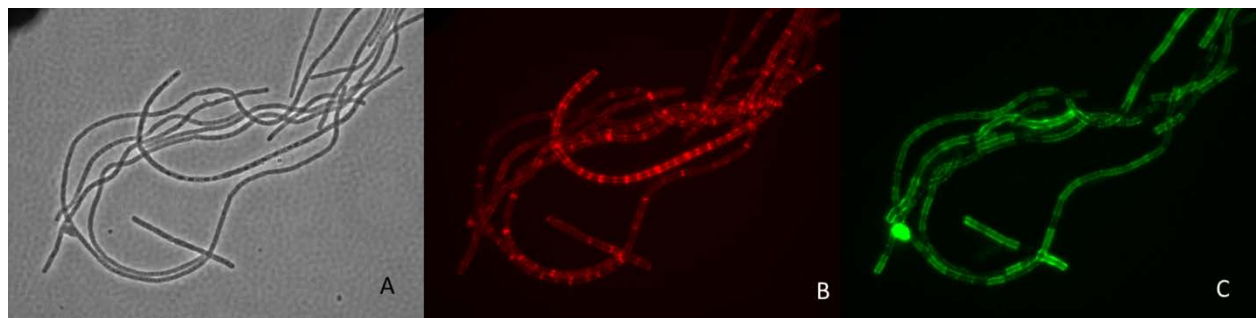
#### Direct agent immunoassays.

Lateral flow immunochromatography assays (LFIA/LFT/LFA), hand-held assays (HHA), "Smart tickets": LFAs are commercially available and potentially useful for direct detection of *B. anthracis* (63, 67). There are two varieties of HHAs developed; a spore based and a clinical, or vegetative cell based (33) assay. The spore-based assay has the inherent problem with the specificity, since antibodies being used may have cross-reactions (false-positives) with other *Bacillus* spp. Development of a more specific spore-based HHA is an effort by several labs but the constraint so far has been development of good antibodies that can be produced in large enough quantities to provide large-scale manufacturability. For the clinical assay, either PA, LF, or capsule antigen is often used. LFAs can detect as little as 25 ng/ml of PA, showing decent sensitivity. For spore based LFAs, in one study of commercial assay kits, all were able to detect *B. anthracis* at  $10^6$  spores while some were able to consistently detect  $10^5$  spores. Sensitivities ranged from 30.43% to 41.6% and a specificity of 75% to 100% (a positive reaction with *B. thuringiensis*) (17, 55).

Enzyme-linked immunosorbent assay (ELISA): ELISAs have been developed for the detection of various components of *B. anthracis*, including spore components, capsule, and the toxins. Like the LFAs, the assays are reasonably fast, sensitive, and specific.

Time-resolved fluorescence (TRF) assay: A TRF assay that measures protective antigen (PA) has been developed for rapid detection of acute cases (22, 53). TRF is falling out of favor due to the difficulty in reagent production and the relatively short shelf-life of the reagents. The assays are fairly specific for clinical use and are useful if encountered.

Direct fluorescent antibody (DFA): The *B. anthracis* DFA specifically and individually targets the unique *B. anthracis* capsule and polysaccharide. The assay is typically quick to perform and interpret but cells must be in the correct phase of growth for optimal reactions. The capsule must be expressed and can be induced with bicarbonate media or increased atmospheric CO<sub>2</sub>. The polysaccharide might be physically covered in later stage growth by capsule or other excretable proteins (S-layer or EA) and care must be taken to prevent or minimize those constituents.



**Figure 13. *B. anthracis* direct fluorescent antibody testing. A) brightfield microscopy B) cell wall polysaccharide (Dylight 594; red) C) capsule stain (FITC; green). This DFA uses a tandem staining where the anti-cell wall are labelled Fab fragments that will penetrate an encapsulated organism and allow simultaneous staining of both components.**

Electrochemiluminescence (ECL): An ECL assay for protective antigen (PA) has been shown to have equivalent sensitivity as culture and nucleic acid amplification (PCR) for the detection of *B. anthracis* infection in non-human primates, yet the ECL assay was available hours before PCR and a full day before conventional culture. Since the assay is PA based, to detect spores in environmental samples, samples traditionally needed to be cultured or 'germinated' (allowing the spore to open and become vegetative) before testing. Germination in heart infusion broth with a mixture of amino acids, adenine, uracil, and salts (HIBAAAUS) for 16 h will generate PA for ECL and other immunoassays. An ECL peptide cleavage assay for lethal factor has also been developed (41). For direct spore detection, a more recent report uses monoclonal antibodies that target *B. anthracis* via BclA with sensitivities down to approximately 1000 spores (19, 20, 27, 49, 57).



Serological testing. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for all serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

Enzyme-linked immunosorbent assay (ELISA): ELISAs can detect antibodies resulting from exposure to *B. anthracis*. An ELISA directed against the protective antigen (PA) has been used in serologic testing for retrospective diagnosis and vaccination verification. Development of measurable antibodies in recent cases required 10 to 16 days after onset of overt disease, but peak IgG levels may not be seen until 40 days after symptom onset. Serum should be collected during acute illness, and 14, 28, 42, and 60 days after onset.

Ascoli test: This assay is an agar gel precipitation test where a piece of hide or tissue is boiled in water and an 'extract' is obtained. The clear fluid is layered on a very narrow application of serum on a agar base. In positive cases, a whitish ring appears at the junction of the two fluids. This test is rarely used but it is provided as a reference to an alternative immunoassay that may be used in some other countries.

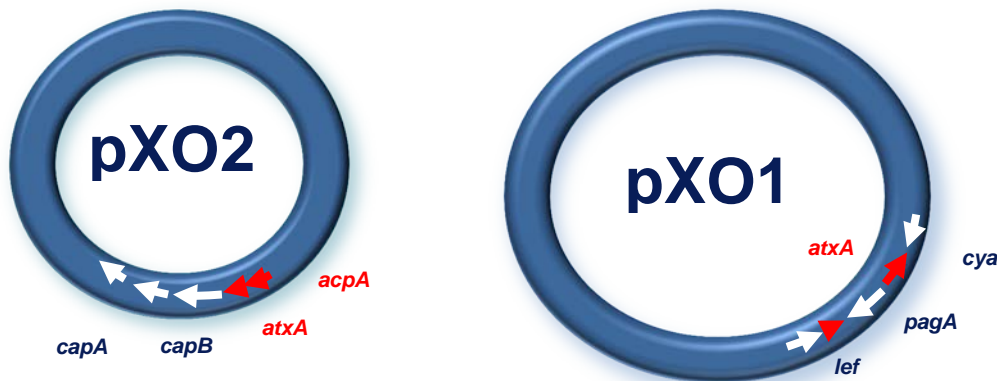
### Nucleic Acid Detection Tests.

There have been a number of polymerase chain reaction (PCR) detection systems have been developed for *B. anthracis* and most are adequate for use. For routine purposes, primers to one of the toxin genes (usually the protective antigen gene) and to one of the capsule formation genes provide a good presumptive diagnosis. A chromosomal marker is often included as a third orthogonal target. PCR analysis has been used solely for analysis of an unnatural outbreak of anthrax (9, 40, 43).

#### Plasmids.

pXO1 (182 kb / 110-Mda / 143 ORFs) contains genes required for synthesis of the anthrax toxin proteins: *cya* which encodes edema factor (EF), *lef* which encodes lethal factor (LF) and *pagA* which encodes protective antigen (PA). Plasmid pXO1 also harbors two trans-acting regulatory genes (*atxA* the toxin gene transactivator), *pagR* (the negative regulator of *pagA*), a gene encoding a type I topoisomerase (*topA*); a resolvase and a transposase, an operon containing three genes (*gerXC*, -A and -B) whose functions appear to affect germination.

Plasmid pXO2 (96 kb / 60Mda /85 ORFs) carries genes required for the synthesis of the poly-



gamma-D-glutamic acid capsule. There are three genes required for capsule synthesis (*capB*, *capC*, and *capA*), a gene associated with capsule degradation (*dep*), and a trans-acting regulatory gene (*acpA*).

The Joint Biological Agent Identification and Diagnostic System (JBAIDS) for the Anthrax Detection System is a fully integrated, FDA approved, in-vitro diagnostic (IVD) system. The intended use statement: The JBAIDS Anthrax Detection System is a real-time PCR test system intended for the qualitative in vitro diagnostic (IVD) detection of target DNA sequences on the pXO1 plasmid (Target 1) and the pXO2 plasmid (Target 2) from *B. anthracis*. The system can be used to test human whole blood collected in sodium citrate from individuals suspected of having anthrax, positive blood cultures, and cultured organisms grown on blood agar plates. The JBAIDS Anthrax target 2 assay is used as a supplementary test only after a positive result with the Target 1 Assay. Results are for the presumptive identification of *B. anthracis*, and are to be used **in conjunction with culture and other laboratory**

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**tests.** In addition, for strains of *B. anthracis* lacking both pXO1 and pXO2, non-JBAIDS primers specific to the S-layer can confirm the presence of *B. anthracis* chromosomal DNA.

The Next Generation Diagnostic System (NGDS) assay for anthracis continues the JBAIDS assays for pXO1 plasmid (Target 1) and the pXO2 plasmid (Target 2) for *B. anthracis*.

### Other Diagnostic Methods.

Dipicolinic acid (DPA) detection: *B. anthracis* spores are packed full of dipicolinic acid (DPA). Molecules that fluoresce when bound to DPA have shown promise in chemically based anthrax detectors .

### Characterization.

Several methods have been tried for characterization of *B. anthracis*, but the closeness of the other species to the genetic makeup of *B. anthracis*, invalidates the use of many of the commonly used systems. Amplified fragment length polymorphism (AFLP) (see Appendix C. Diagnostic Technologies for a method description) was one of the first methods to try to differentiate the *Bacillus* through genetic methods. AFLP on *Bacillus* demonstrated that *B. anthracis* strains showed very little variability among different isolates (26) but there was a significant difference between *B. anthracis* strains and the other *Bacillus* species (24, 29-31, 60).

From the AFLP work, analysis of variable number tandem repeat (VNTR) sequences was developed. Short nucleotide sequences that are repeated multiple times, often vary in copy number, creating length polymorphisms that can be detected easily by PCR using flanking primers. VNTRs appear to contain greater diversity and, hence, greater discriminatory capacity than many other molecular typing systems. Many bacteria have VNTRs, although development of the PCR primers for these markers is specific to each pathogen .

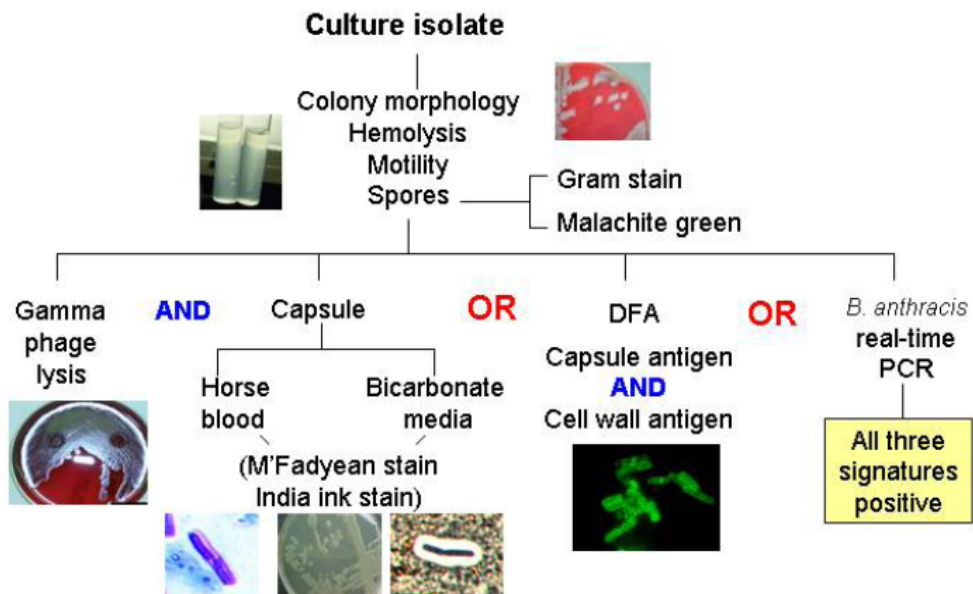
From the VNTR work, "Canonical SNPs" (canSNPs), where "rare" single nucleotide polymorphisms (SNPs) was developed. Their presence and state (nucleotide difference) is useful for identifying long branches or key phylogenetic positions . This is the current method for characterization of *B. anthracis* and a number of other biothreat bacterial agents.

### Confirmation.

Note: For a general discussion on DOD CONOPS for confirmation, see Appendix D).

Presumptive identification of a biological threat agent is achieved by the detection of a biological marker using a single test methodology (for example, hand-held assay (HHA)). Presumptive diagnosis can also be made by Gram, Wright, Giemsa or Wayson stain of blood, sputum, cerebrospinal fluid, or lymph node aspirates.

Definitive identification or confirmation of *B. anthracis* requires culture of the organism and application of appropriate confirmatory tests by the civilian community. Culture can be a DOD method but is currently not a requirement, unfortunately. Culture of the organism can reduce false-positive results from non-specific environmental interference and allows the evaluation of false-negative results in the context of the broad array of testing performed. Definitive identification or confirmation testing is performed at sanctioned reference laboratories, including reference laboratories of the CDC Laboratory Response Network (LRN). Specific LRN protocols and reagents are proprietary but any definitive identification or confirmation typically follows the following general scheme.

***B. anthracis* Culture Identification**

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## HIBAAAUS germination medium

1. Heat-inactivate horse serum (GIBCO-Grand Island, NY) at 56°C for 30 min to destroy complement. Centrifugation at 10,000 X g for 15 min and filter the supernatant through a 0.2 µm Millipore low-protein binding-filter to remove particulate matter.

2. Prepare beef heart infusion (Difco, Detroit, Mich.) at 2.5% w/v and fortify with a mixture of amino acids, adenine, uracil, and salts (AAAUS). AAAUS represents a portion of the components of the defined medium formulation described by Ristoph and Ivins (48) for optimal anthrax toxin production. Make the AAAUS mixture as a 40 X concentrated stock so that when diluted in medium, the following component concentrations (mg/ml) will be obtained:

- L-tryptophan, 35;
- glycine, 65;
- L-cystine, 25;
- L-lysine, 230;
- L-valine, 173;
- L-leucine, 230;
- L-isoleucine, 170;
- L-threonine, 120;
- L-methionine, 73;
- L-aspartic acid, 184;
- sodium L-glutamate, 612;
- L-proline, 43;
- L-histidine-hydrochloride, 55;
- L-arginine-hydrochloride, 125;
- L-phenylalanine, 125;
- L-serine, 235;
- L-thiamine-hydrochloride, 1.0;
- CaCl<sub>2</sub>·2H<sub>2</sub>O, 7.4;
- MgSO<sub>4</sub>·H<sub>2</sub>O, 9.9;
- MnSO<sub>4</sub>·H<sub>2</sub>O, 0.9;
- uracil, 1.4;
- adenine sulfate, 2.1.

3. Induce capsule formation in the fortified beef heart infusion by adding sodium bicarbonate to 0.8% and heat inactivated horse serum to 50%. Sterile 8% sodium bicarbonate is diluted 1:10 into the medium to give a final concentration of 0.8%.

4. If required, the medium may be lyophilized and refrigerated for long-term storage. When lyophilized, the medium can be reconstituted with a liquid spore suspension.

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# *Yersinia pestis*

## OVERVIEW

*Yersinia pestis* is a rod-shaped, non-motile, non-sporulating, gram-negative bacterium of the family *Enterobacteraceae*. It causes plague, a zoonotic disease of rodents (e.g., rats, mice, ground squirrels). Fleas that live on the rodents can transmit the bacteria to humans, who then typically develop the bubonic form of plague. The bubonic form may progress to the septicemic and/or pneumonic forms. Pneumonic plague would be the predominant result of purposeful aerosol dissemination. All human populations are susceptible. Recovery from the disease is followed by temporary immunity. The organism remains viable for a long time in many different matrices: water, moist soil, grains, dry sputum, flea feces, and buried bodies. At near freezing temperatures, *Y. pestis* will remain alive for months to years, but it is killed by 15 min of exposure to 55°C or several hours of exposure to sunlight.

*Y. pestis* is a recently evolved clone of *Y. pseudotuberculosis* (1). *Y. pestis* belong to a smaller group of organisms than *B. anthracis*, but is much more difficult to identify. *Y. pestis* have several plasmids that confer various virulence traits and are useful diagnostic assay targets, but the plasmids are promiscuous and can be found in non-*Y. pestis* causing the potential for false-positive assays. Capsule (F1) is a good marker for the diagnosis of *Y. pestis* but does not get produced at the optimal growth temperature for *Y. pestis* (28°C) but instead is produced at 35-7°C making this marker less reliable for environmental *Y. pestis* detection. Immunoassay and nucleic acid assays are available for diagnostics but confirmation of *Y. pestis* is done using phage on cultural growth.

## ORGANISM INFORMATION

### Taxonomy Information.

*Y. pestis* evolved from *Y. pseudotuberculosis* about 1.5-20 thousand years ago (1) so they are very similar organisms. *Y. pestis* has both gained new genetic information not shared by *Y. pseudotuberculosis*, and lost functions that its progenitor has retained.

Presently, 18 species are included in the genus *Yersinia*; however, only three (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) have been unquestionably shown to be human pathogens. One species, *Y. ruckeri*, a fish pathogen and not known to cause human infection, may be moved to a new genus.

Classic taxonomy of *Y. pestis*.

Subspecies taxonomy and a biovar taxonomy are both used.

Subspecies taxonomy (favored by Russian and Chinese researchers)

*Yersinia pestis* subspecies *pestis*; classical strains causing lethal disease

*Yersinia pestis* subspecies *microtus*; only cause disease in some rodents (73);  
'pestoides' strains (2).

Biovar taxonomy

Biovar *antiqua* (Africa, southeastern Russia, central Asia; thought to be the cause of the first pandemic) are glycerol positive, nitrate positive strains.

Biovar *medievalis* (Caspian Sea; thought to be the cause of the second pandemic) are glycerol positive, nitrate negative strains.

Biovar *orientalis* (Asia, Western Hemisphere; cause of the third pandemic) are glycerol negative, nitrate positive strains.

Currently the pandemic strain.

Classical biovars do not correlate directly with classification based on genotypic markers (2).

Biovar *microtus* or 'pestoides' Evidence of early endemic forms of *Y. pestis* remains in the ancient plague reservoirs of Russia. These "pestoides" isolates differ from the epidemic forms of the species in that pPCP is not required for tissue invasion (57, 69). The pestoides strains therefore refer to enzootic variants that remain in ancient reservoirs where infection is limited to hamsters, gerbils, true mice, and rats, and many other relative rodents. For humans, they are typically considered attenuated strains of *Y. pestis* (53).

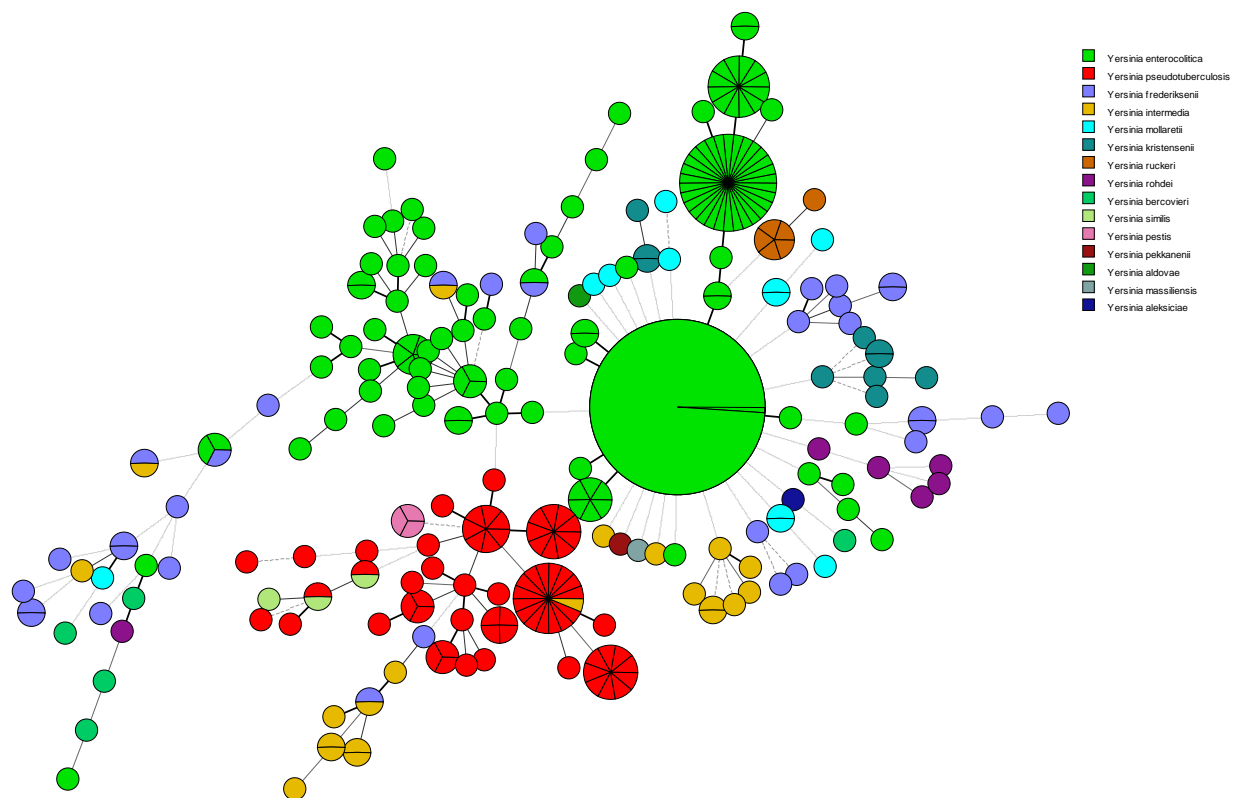


Figure 14. A minimum spanning tree representing the diversity of *Yersinia* spp. as determined by multilocus sequencing. Note the placement of the *Y. pestis* branch off the *Y. pseudotuberculosis* branch. Node sizes are a reflection of the data used to create the comparison.

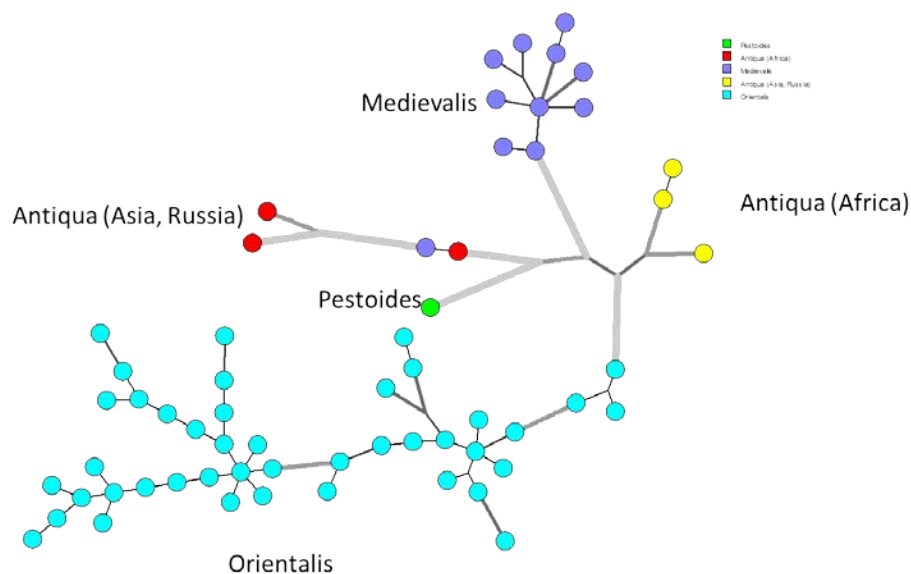


Figure 15. Minimum spanning tree construct of *Y. pestis* VNTR genotypes (35). The biovar nomenclature demonstrates the fidelity of the data clustering.

Virulence Factors.

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The main virulence factors of *Y. pestis* are encoded on three plasmids (18, 19, 47, 46) and chromosomally (28, 42, 51).

	Other names	Size		Function
pPCP	pPst, pPla, pPCPI	9.6 kb	Pestis only	<i>pst/psn</i> -pesticin, <i>pla</i> -plasminogen activator, <i>pim</i> -pesticin immunity protein
pMT	pYmt pFra,	101 kb	Pestis only	<i>Caf</i> -F1 capsule, <i>ymt</i> -murine toxin
pCD/pYV	pCad, pYV, pYVe8081	70 kb		LCR – low calcium response, <i>Yops</i> -virulence proteins associated with adhesion, injection into cell, inhibition of eukaryotic cell functions;

A small **9.6 kb plasmid (pPCP)** encodes the three gene set; **pesticin**, **coagulase**, and **plasminogen activator**. Pesticin is a unique *Y. pestis* bacteriocin that is active against serotypes IA and IB cells of *Y. pseudotuberculosis* and other bacteria. Two genes are required, *pst*-pesticin and *psn* – pesticin receptor gene. Pesticin is a bacterial homolog of phage lysozymes. The activity of the protein is in the periplasma leading to hydrolysis of peptidoglycan and cell lysis. Pesticin immunity protein (*pim*) produces a 141 amino acid polypeptide that protects *Y. pestis* from the action of its own pesticin. The protein also localizes in the periplasma and blocks the action of pesticin. Of the three structural genes present in pPCP, only *pla* (34.6-kD protein) encodes a virulence factor. *Pla* is a protease that activates plasminogen, causes blood clots at the bite wound to dissolve, and allows the initial dissemination of the bacteria to the lymph nodes (55, 60).

A 70-kb plasmid (pCD; **calcium dependence**) is necessary for human pathogenicity. It is also present in *Y. enterocolitica* and *Y. pseudotuberculosis* (where it is sometimes called pIB1 or pYV) but different gene orders exist on the plasmids. The plasmid encodes the *Yersinia* outer protein system (*Yops*) and the **low calcium response (LCR)**, along with a cell surface adhesion system (*yadA*; *Yersinia* adhesion A). The low calcium response consists of a series of genes responsible for calcium uptake and anti-phagocytic activity (antigen LcrV). The LCR system exists in both *Y. pestis* and *Y. pseudotuberculosis* and is not specific, but the loss of the LCR function greatly attenuates the organisms. Absence LCR plasmid (pCD1) is sufficient to exempt the strain from select agent (BSAT) status. The three pathogenic species share the *Yops*, which are secreted and translocated by a type III secretion system (*Ysc*). The type III secretion system (TTSS) helps to undermine the host immune system. Loss of the plasmid results in decreased pathogenicity and an inability to cause disseminated disease. Several *Yops* proteins are currently known, *YopH*, *YopM*, *YopB*, *YopD*, *YopN*, *YopE*, *YopK*, and others may exist (16).

A 100- to 110-kb plasmid (pMT) encodes the murine toxin *Ymt* and the **F1 capsular protein** (*Caf1*). pMT in its entirety, can readily integrate into the chromosome thus potentially existing as a second high-pathogenicity island. This is probably due to the 18-kb operon for *caf1* and attendant functions being linked to bacteriophage homologs. The plasmid encodes not only structural genes for *caf1* and murine toxin (MT), but also has genes for a number of common functions concerned with normal vegetative growth. At 37°C *Y. pestis* expresses a **capsule**-like antigen called fraction 1 (F1). F1 is encoded by the *caf1* gene. F1 is a surface polymer composed of a protein subunit with a molecular mass of 15.5 kD. This determinant confers resistance to uptake by certain phagocytes, especially neutrophils and monocytes, but it evidently is ineffective against fixed macrophages lining the capillary network of liver and spleen (26, 50, 68). *Caf1* is required for virulence in guinea pigs but not necessary for virulence in man or mice (71). In addition to *caf1*, *caf1M* is a chaperone protein; *caf1A* is involved in capsule anchoring, and *caf1R*, an activator protein (29, 36, 49, 50). **Murine toxin** (*ymt*) is another protein required for survival within the otherwise hostile environment of the flea midgut and indicates that this toxic is required for colonization (as opposed to blockage) of the flea midgut.

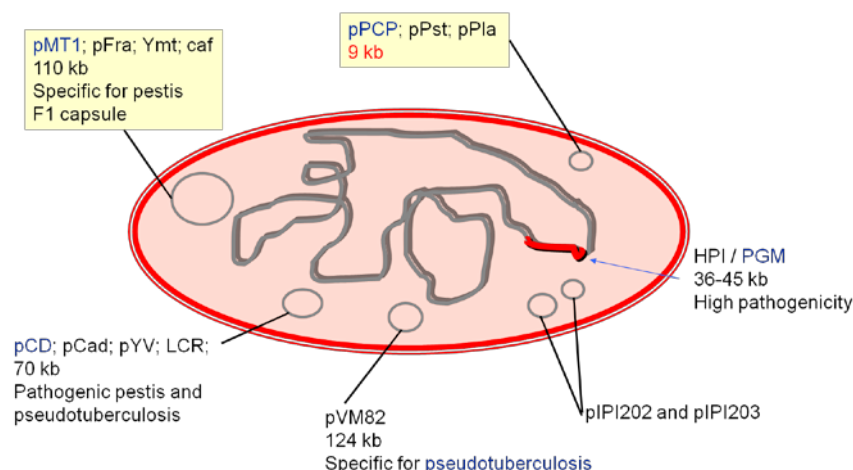


Figure 16. Cartoon of the plasmids in *Yersinia* and the high pathogenicity island. pMT and pPCP are considered *Y. pestis* specific plasmids, but other organisms have been known to harbor these or similar plasmids. Loss of pCD or PGM loci will attenuate the organism sufficient for CDC to exempt the agent from BSAT controls.

At least one chromosomal genetic element, the *pgm* locus is composed of two distinct parts: a pigmentation segment and a **high-pathogenicity island (HPI)** segment. The pigmentation segment carries the *hms* genes and the HPI carries genes involved in iron acquisition (yersiniabactin biosynthetic gene cluster) (8, 9, 11, 59). The HPI contains at least three systems involved in the iron uptake that is required for virulence. These systems consist of the siderophore (yersiniabactin)-dependent process, the linked hemin storage function (*hms* locus), a siderophore-independent ferric uptake (*yfu*) system, and a siderophore-independent transporter (*Yfe*) system.

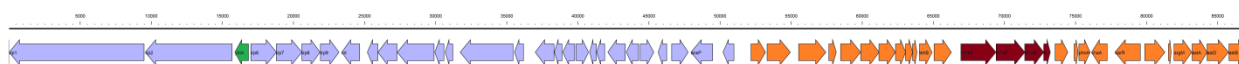


Figure 17. Organization of the chromosomal *pgm* locus. Insertion elements flank the region and account for the high frequency of deletion. The HPI portion is indicated in blue with one of the *ybt* genes (green) for yersiniabactin synthesis. The pigmentation segment (orange) and the *hms* genes (red) are the hemin-binding components. Partial sequence from position 2146470 to 2233661 from NC\_003143.

Deletion of the **entire** *pgm* locus significantly reduces virulence to the point where the CDC exempts *pgm* negative strains from select agent control. *Pgm*<sup>-</sup> strains, such as the EV 76 strain, have been used for years as live human vaccines with no significant plague-associated illness. The *hms* genes are essential for the blockage of the flea proventriculus and the ability to absorb hemin and the dye Congo red. At temperatures of 26°C or less, *Y. pestis* colonies are pigmented (*Pgm*<sup>+</sup>) on Congo red medium (63). PCR and/or Southern blot analysis will be required to ensure that "*Pgm*<sup>-</sup>" derivatives have undergone a complete deletion rather than a mutation in hemin storage genes (*hms*), which can also causes loss of Congo red binding; the most common characteristic used to evaluate the pigmentation phenotype.

In 2009, Professor Malcolm Casadaban passed away (5). Professor Casadaban had been working on a live attenuated vaccine strain of *Y. pestis* KIM D27 at the University of Chicago. *Y. pestis* KIM D27 is a strain of plague, which was approved by the Centers for Disease Control and Prevention (CDC) as safe for researchers due to attenuation of virulence. *Y. pestis* KIM D27 is a vaccine strain (live-attenuated vaccine strain) and exempted from select agent controls in 2008 because it was *Pgm* negative. People with chronic conditions, however, are at higher risk and for some bacterial infections: conditions like hereditary hemochromatosis and hemosiderosis (which Professor Casadaban had) where the inherent iron build up compensates for the *Y. pestis* lack of an iron uptake system! **Hence, *pgm* negative strains are attenuated, NOT AVIRULENT!**

Determinate	Location	Gene	function	Diagnostic use	Expression	<i>Y. pestis</i>	<i>Y. pseudotuberculosis</i>	<i>Y. enterocolitica</i>
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<b>Pesticin</b>	plasmid pPCP; 9.5 kb	<i>pst</i>		detection of pathogenic strain:		+	=	
<b>Plasminogen activator</b>	plasmid pPCP; 9.5 kb	<i>pla</i>	Fibrinolysis Coagulase	detection of pathogenic strain:	37°C 28°C	+	=	
<b>Capsule (fraction 1;F1)</b>	plasmid pMT; 110 kb	<i>caf1</i>	Antiphagocytic	detection of pathogenic strain:	37°C	+	=	
<b>Murine toxin</b>	plasmid pMT; 110 kb	<i>ymt</i>	Phospholipase D; erythrocyte lysis in flea proventriculus	none	30°C	+	=	=
<b>Pigmentation</b>	Chromosome HPI/pgm	<i>hms</i>	Iron or hemin binding, flea blockage	Congo red or hemin binding	26 to 30°C	+	+	
<b>Flagella</b>	Chromosome	many	Motility	Motility	22 to 30°C	=	+	+
<b>Yeisinabactin (siderophore)</b>	Chromosome HPI/pgm	<i>ybt</i>	Iron uptake	Pesticin sensitivity siderophore production; Congo red phenotype; detection pathogenic /select agent strain	37°C	+	+	+
<b>Invasin</b>	Chromosome	<i>invA</i>	Adhesin	detection of pathogenic strain	26°C (pH 8) 37°C (low pH)	=	=	+
<b>Attachment invasion-locus protein (Ail)</b>	Chromosome	<i>ail</i>	Adhesin	detection pathogenic strain	37°C	=	+	+
<b>Enterotoxin</b>	Chromosome	<i>yst</i>	Heat,stable enterotoxin	detection of Y. enterocolitica; species specific	26°C	=	=	+
<b>Low calcium response (LCR)</b>	Plasmid, 70 kb	<i>lcr</i> (ysc)	Effectors of Yap secretion	detection pathogenic /select agent strain	37°C	+	+	+
<b>Yaps,</b>	Plasmid, 70 kb	<i>yop</i> genes	secreted and translocated by pestin type III secretion apparatus Antiphagocytic	Detection and differentiation	37°C	=	+	+
<b>Membrane protein</b>	Plasmid, 70 kb	<i>yadA</i>	Adhesion to cells and extracellular matrix proteins	Detection and differentiation	37°C	=	+	+
<b>VirF protein</b>	Plasmid, 70 kb	<i>virF</i>	Transcriptional, activator for expression of Yops	detection of pathogenic strain	37°C	+	+	+

Epidemiology and Endemic Areas.



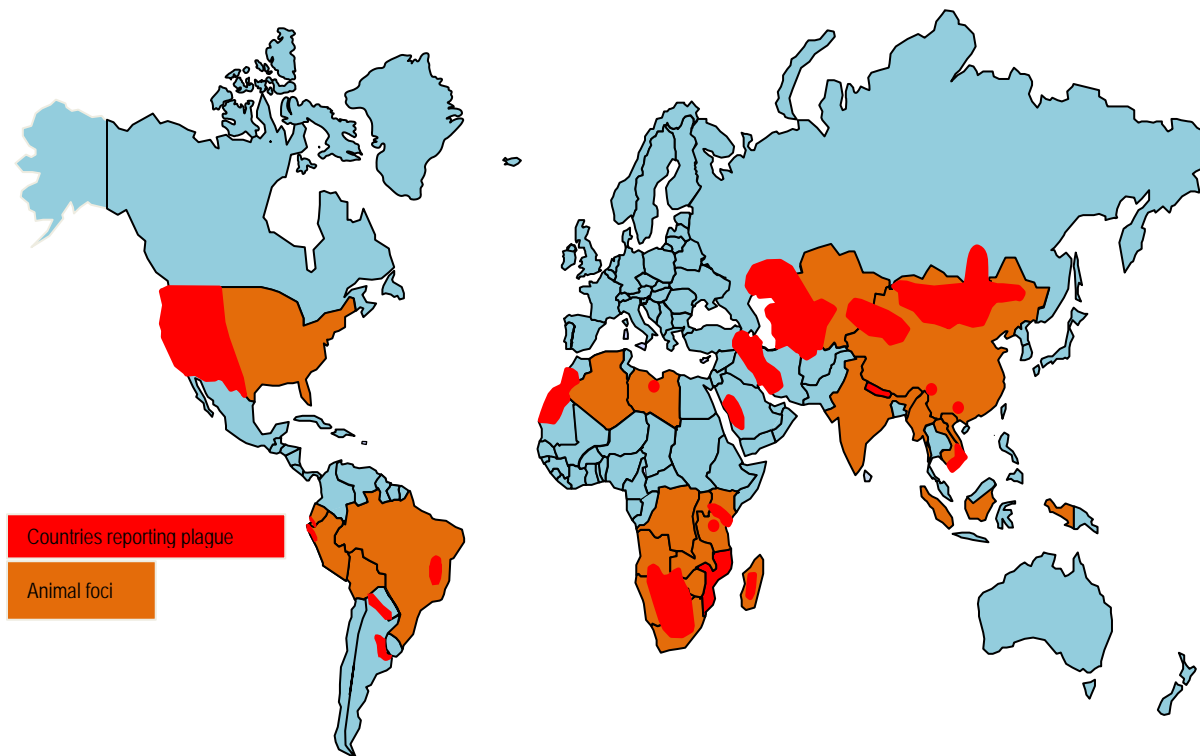


Figure 18. Global distribution of *Y. pestis*.

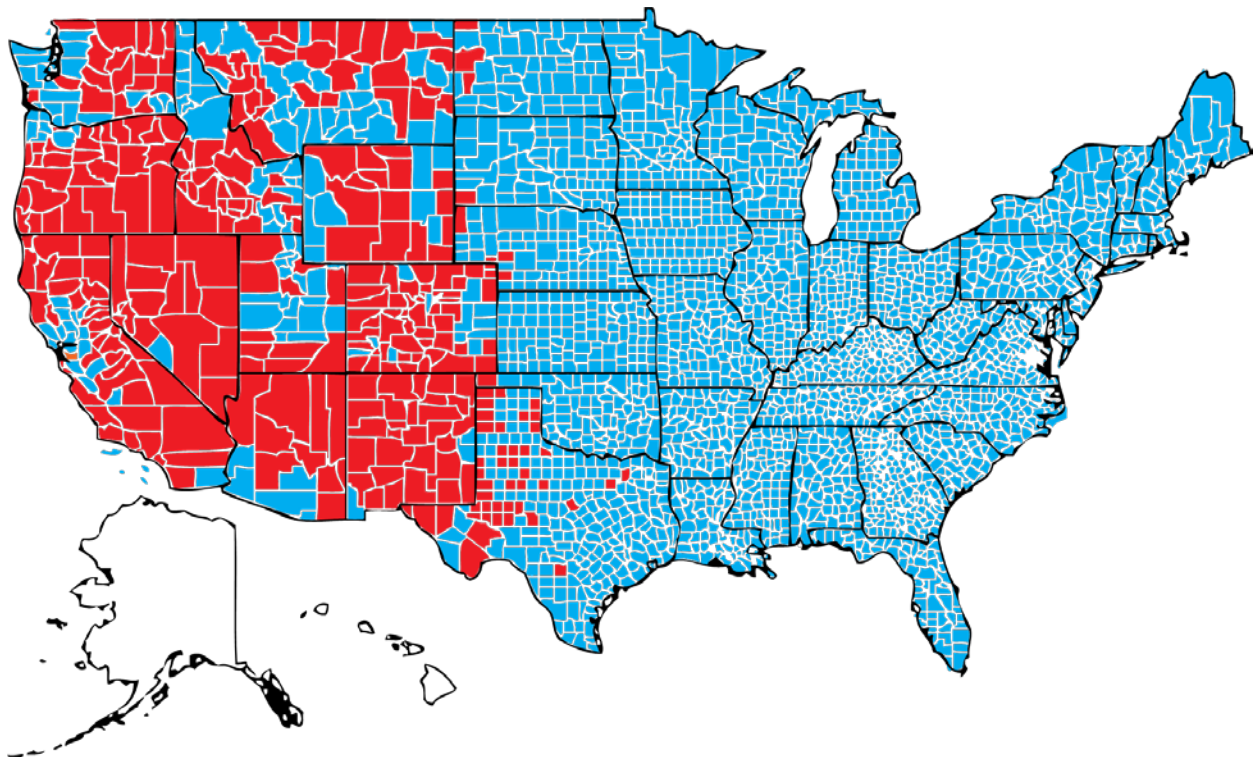


Figure 19. Distribution of *Y. pestis* in the US. Red counties have been reported with historical plague cases.

## LABORATORY DIAGNOSTICS

### Biosafety Information.

Biosafety in Microbiological and Biomedical Laboratories (BMBL) Containment Recommendations (5th Edition): Biosafety level (BSL)-2 practices, containment equipment, and facilities are recommended for all activities involving the handling of potentially infectious clinical materials and cultures. In addition, because the infectious dose is so small, all work, including necropsies of potentially infected animals should be performed in a biological safety cabinet. Special care should be taken to avoid generating aerosols or airborne droplets while handling infectious materials or when performing necropsies on naturally or experimentally infected animals. Gloves should be worn when handling potentially infectious materials including field or laboratory infected animals. BSL-3 is recommended for activities with high potential for droplet or aerosol production, and for activities involving large scale production or high concentrations of infectious materials. Resistance of *Y. pestis* strains to antibiotics used in the treatment of plague should be considered in a thorough risk assessment and may require additional containment for personal protective equipment. For animal studies, a risk assessment that takes into account the animal species, infective strain, and proposed procedures should be performed to determine if animal biosafety (ABSL)-2 or ABSL-3 practices, containment equipment, and facilities should be employed. BSL-3 facilities and arthropod containment level 3 practices are recommended for all laboratory work involving infected arthropods.

### Diagnostic Information.

#### General Information.

*Yersinia pestis* is a gram-negative, non-motile, capsule producing, non-spore forming, rod-shaped coccobacillus, facultatively anaerobic, with a width of 0.5-0.8 µm and a length of 1-3 µm. It causes the disease called "plague" and is principally a zoonotic disease with human infections being incidental. Human *Y. pestis* infection takes three main forms: pneumonic, septicemic, and bubonic plagues.

#### Staining.

NOTE: Consistent, striking bipolar safety-pin morphology of the bacilli is characteristic of *Yersinia*, *Pasteurella* spp. and other organisms. Bipolar appearance, which is due to the presence of a central vacuole, is not unique to *Y. pestis* but is typically an indicator. Bacterial cells picked from freshly passaged agar/broth growth tend to exhibit very little bipolarity, because the cells are too small; however, upon prolonged incubation, the cells would be more likely to exhibit the characteristic bipolar safety-pin shapes. The Wright-Giemsa stains are the most reliable for accurately highlighting the bipolar staining characteristics of *Y. pestis*, whereas the Gram stain may not. The most suitable materials for differential staining include a bubo aspirate, sputum, blood smears, and tissues (lung, spleen, liver).

Gram Stain: Stained specimens containing *Y. pestis* often reveal plump, gram-negative rods, 1-2 micrometer X 0.5 micrometer, that are seen mostly as single cells or pairs and short chains. The Gram stain can be used as supportive, but not confirmatory evidence of *Y. pestis* infection.

Wright-Giemsa: There are related stains known as the buffered Wright stain, the Wright-Giemsa stain, and the buffered Wright-Giemsa stain, and specific instructions depend on the solutions being used, which may include eosin Y, Azure B, and methylene blue (some commercial preparations combine solutions to simplify staining). The May-Grünwald stain, which produces a more intense coloration, takes a longer time to perform. Wright's stain is a histologic stain that facilitates the differentiation of blood cell types. It is used primarily to stain peripheral blood smears and bone marrow aspirates which are examined under a light microscope. The stain, a modification of the Romanowsky stain, is used to distinguish easily between blood cells, but visualizes any bacteria that may also be present.

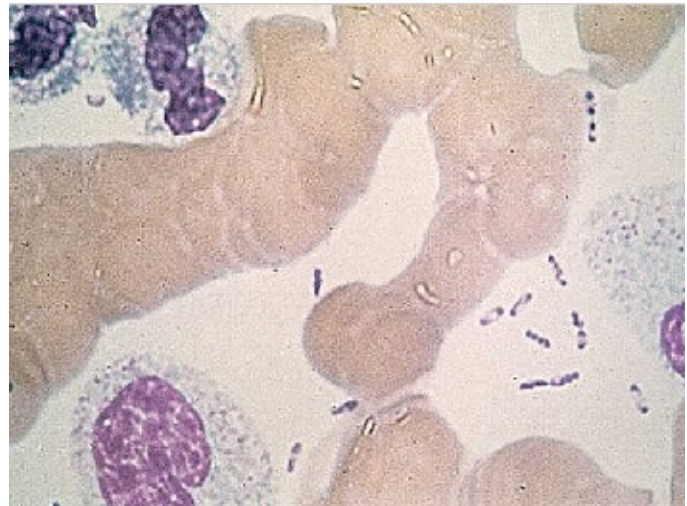


Figure 20. Giemsa stain of *Y. pestis*. Note the characteristic "safety pin" appearance of the bacteria.



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Wayson stain: Another adaptation of the basic fuchsin-methylene blue, ethyl alcohol-phenol microscopic staining procedure, is a modified methylene blue stain that is good for visualizing *Y. pestis*. With this stain, *Y. pestis* appears purple with a characteristic safety-pin appearance.

Direct immunofluorescence assays (DFA): Direct immunofluorescence assays (DFA) for cell-wall-associated polysaccharide and capsule produced by vegetative cells (demonstration of both antigens provides confirmatory identification). This used to be included in confirmatory testing. (Discussed more under "Immunoassay" section)

#### Metabolic Information

General: Members of the genus *Yersinia* are non-spore-forming, gram-negative, rod-shaped or coccoid cells 0.5 to 0.8  $\mu\text{m}$  in width and 1 to 3  $\mu\text{m}$  in length with single cells or short chains. Except for *Y. pestis*, which is non-motile, the other species are motile at 22-30°C but not at 37°C; motile cells are peritrichously flagellated. *Yersinia* grow under aerobic and anaerobic culture conditions and are facultative anaerobes, non-sporulating, and are non-lactose fermenters. *Yersinia* are catalase-positive, oxidase- and urease-negative (rarely, strains may be urease-positive), produce an alkaline slant/acid butt (K/A) on triple sugar iron agar (TSI) without gas or  $\text{H}_2\text{S}$ . They are relatively inert in other biochemical tests. *Y. pestis* lacks many enzymatic functions such as adenine deaminase, aspartase, ornithine decarboxylase, glucose-6-phosphate dehydrogenase, and urease activities. Other than glucose and mannitol, it also lacks the ability to ferment most other carbohydrates. Therefore *Y. pestis* has special nutritional requirements for L-valine, L-methionine, L-phenylalanine, and glycine or L-threonine. These requirements limit its survival outside the mammalian or flea host but do not present an issue for routine culturing.

On all solid media, *Y. pestis* grows as pinpoint colonies after 24 h incubation at 35°C. The morphology becomes more typical after 48 h, when the organisms are nonhemolytic on blood agar, smooth or mucoid on BHI agar, and lactose negative on MacConkey agar. Virulence plasmids are easily lost with subculture at 35-37°C. Virulent strains grown on nutrient agar at 25-30°C frequently dissociate into small (plasmid-positive) and large (plasmid-negative) colonies. Less common phenotypic tests for recognizing virulent organisms are the uptake of Congo red at temperatures below 30°C, and calcium dependency at 37°C on magnesium oxalate agar (64).

Optimal temperature: 25-28°C.

Upper temperature: 45°C.

Lower temperature: 0°C.

Optimal pH 7.2 to 7.6

Upper pH: pH 9.6

It is generally recognized that the doubling times of *Y. pestis* are significantly greater than those observed for typical enteropathogenic *Yersinia* in media supplemented with  $\text{Ca}^{2+}$ . This difference largely disappears, however, if the concentration of atmospheric  $\text{CO}_2$  is brought to 10%, whereupon single cells of *Y. pestis* can form visible colonies at 37°C on enriched solid medium in 24 h rather than the usual 2 days. The concentration of metabolic  $\text{CO}_2$  generated in suitably enriched liquid media is sufficient to permit cells of *Y. pestis* to achieve doubling times during the mid-logarithmic growth phase of about 70 min at both 26° and 37°C. This value approximates that observed for enteropathogenic *Yersinia* cultivated in the same medium (12). That *Y. pestis* grow more rapidly at 26-28°C than at 35-37°C is actually incorrect and reflect lack of knowledge of the nature of the sluggish regulation, the nutritional requirement for  $\text{Ca}^{2+}$ , and the stimulatory effect of  $\text{CO}_2$ .

For species identification, fermentation of glycerol and reduction of nitrite are key factors. Three biovars are currently recognized: Antiqua, Medievalis, and Orientalis. Differentiation of these biovars is based on nitrite reduction and utilization of glycerol.

Antiqua ferments glycerol and reduces nitrite.

Medievalis lost the ability to reduce nitrate but is glycerol positive.

Orientalis cannot do either.

Pestoides ferment glycerol and reduce nitrite.

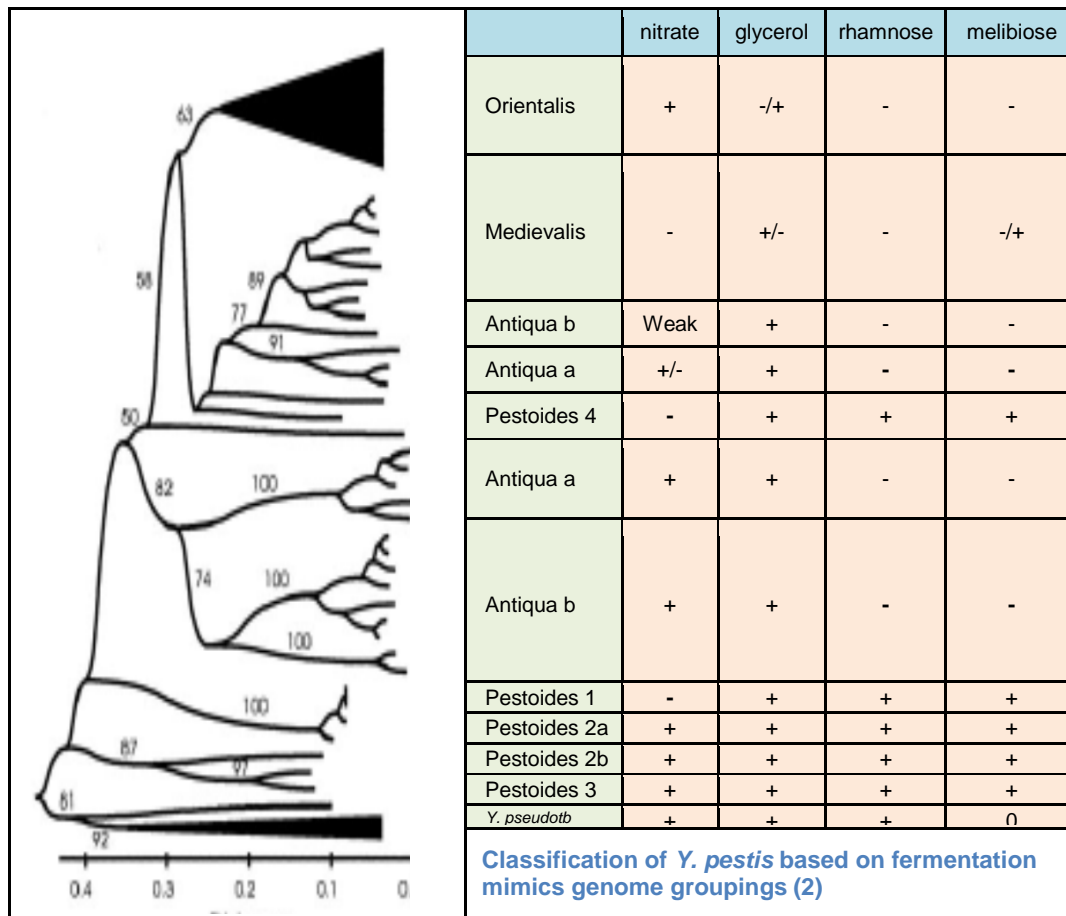
Fermentation of rhamnose and melibiose: To ensure differentiation of the pestoides strains from virulent *Y. pestis* strains, fermentation of rhamnose and melibiose can be used.

Antiqua does not ferment either.

Medievalis can occasionally ferment melibiose.

Orientalis does not ferment either.

Pestoides ferments both rhamnose and melibiose.



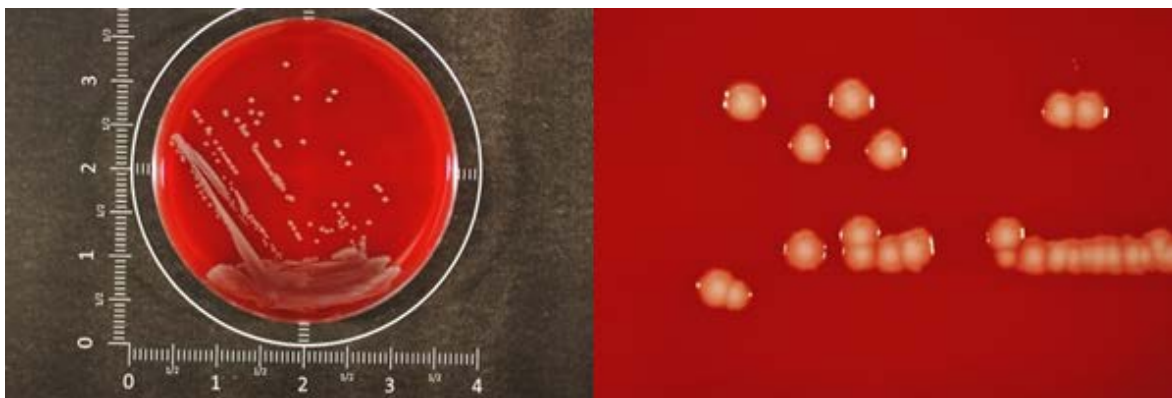
#### General culturing Information

It is generally recognized that the doubling time of *Y. pestis* strains are significantly greater than those observed for typical enteropathogenic *Yersinia* spp. This difference largely disappears, however, if the concentration of atmospheric CO<sub>2</sub> is brought to 10% whereupon single cells of *Y. pestis* can form visible colonies at 37°C on enriched solid medium in 24 h rather than the usual 2 days. The concentration of metabolic CO<sub>2</sub> generated in suitably enriched liquid media is sufficient to permit cells of *Y. pestis* to achieve doubling times during the mid-logarithmic growth phase of about 70 min at both 26° and 37°C.

**Nutrient agar (NA).** *Y. pestis* grows well on nutrient agar. Because of its slower growth, *Y. pestis* may be quickly overwhelmed by contaminants. Colonies are small, delicate, and transparent after 24-48 h of incubation.

**Nutrient broth (NB).** *Y. pestis* grows well in nutrient-rich broths such as brain heart infusion (BHI), trypticase soy, or nutrient broth. The organisms exhibit a characteristic growth formation in broth, whose appearance may be used as an aid to its identification. Because of its slower growth, *Y. pestis* may be quickly overwhelmed by contaminants. Broth cultures can be described as suspended flocculent or crumbly clumps ("stalactites"). These clumps are visible at the side and bottom of the tube. Longer incubation will result in the clumps falling to the bottom of the tube and loss of the characteristic formation.

Sheep blood agar (SBA): *Y. pestis* organisms are not fastidious and will grow well on any nutrient medium including SBA. *Yersinia* spp. grow slower than many bacteria at 37°C, but at 28°C they will grow faster than most. Enrichment of medium with 6% sheep red blood cells instead of the standard 5% provides more nutrition and shortens the incubation period. Even though *Y. pestis* may grow faster at 28°C, a plate should also be incubated at 37°C since diagnostic tests for plague depend primarily on



expression of the temperature-regulated F1 antigen. Primary plates should be held for 5 days and held for up to 7 days if the patient has been treated with antibiotics. *Y. pestis* colonies are gray-white, translucent, usually too small to be seen as individual colonies at 24 h. After incubation for 48 h, colonies are about 1-2 mm in diameter, gray-white to slightly yellow, and opaque. Under 4X enlargement, after 48-72 hour of incubation, colonies have a raised, irregular "fried egg" appearance, which becomes more prominent as the culture ages. Colonies also can be described as having a "hammered copper," shiny surface. There is little or no hemolysis of the sheep red blood cells.

#### Notes:

- Sheep blood agar (SBA) plates are typically used as the standard solid medium for the isolation and culture of *Y. pestis*. If SBA plates are not available, other general solid medium such as brain heart infusion agar, nutrient agar or trypticase soy agar may be used, though growth of the organism will be slower and colonies will be smaller.
- Highly passaged and laboratory-adapted strains grow faster and colonies are larger.
- With some samples, enriched by inoculation into mice may be needed.

#### Selective Culturing Information

MacConkey agar. This selective and differential medium is commonly used in many laboratories. While not specifically selective for *Yersinia* spp., *Y. pestis* grows as a typical lactose negative colony (flat, colorless, or pale pink) at 1-2 mm diameter after 18-24 h at 35-37°C.



Figure 23. *Y. pestis* on MacConkey agar at 48 hrs.

Yersinia Selective Agar (=CIN Agar, Cefsulodin-Irgasan-Novobiocin Agar): This is a selective differential medium developed for the isolation of *Y. enterocolitica*. *Y. pestis* grows on this medium, but is slightly inhibited thus this medium is typically not recommended for *Y. pestis* (Bockemühl 2003). *Y. enterocolitica* and *Y. pestis* colonies have deep-red centers surrounded by a transparent, pale border



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giving the appearance of a "bull's-eye" after 24 h of incubation. After 42 to 48 h of incubation, they are often completely pink. *Y. pseudotuberculosis* usually lacks the transparent zone around the colonies. *Aeromonas* spp. produce paler colonies, which also have a rose to red center. *Enterobacteriaceae* other than *Yersinia* may grow on this medium, especially *Serratia* and *Citrobacter* species. *Serratia* and *Citrobacter* cannot always be reliably differentiated from *Yersinia* by colony morphology alone. A different formulation of only 0.004 g of cefsulodin per liter medium instead of 0.015 g of cefsulodin has been recommended for recovery of *Y. pestis* (7, 58).

**BIN selective agar:** It was purportedly found that BIN agar is more efficient in supporting colony formation and recovery of *Y. pestis* than are the conventional semisolid media MacConkey agar and *Yersinia*-selective agar (cefsulodin-irgasan-novobiocin), though these media has not been widely employed. Gray *Y. pestis* colonies of ~2 mm in diameter require 2 days to develop. The authors reported that white *Vibrio cholerae*, yellow-green *Pseudomonas aeruginosa*, and red-pigmented *Serratia marcescens* colonies could be observed after 1 day, whereas *Enterobacter faecalis* growth was very slow, and colony diameters were less than 0.5 mm after 3 days of incubation. No further strains were reported (6).

***Y. pestis* chromogenic agar (YpCM; R & F Laboratory Products):** *Y. pestis* colonies appear blue-black, raised, 0.5 to 2.0 mm in diameter, with or without a clear ring and precipitate. Other colony types appearing on this plating medium that are not presumptive *Y. pestis* strains, are blue-green with and without a precipitate, green with and without a precipitate, yellow-green with and without a precipitate, yellow with and without a precipitate, clear and white.

#### Diagnostic Tests.

*Yersiniae* are metabolically more active at 25-30°C than at 35-37°C. Therefore, incubation at lower temperatures is strongly advised for most biochemical tests, but many schemes use the more common 35-37°C incubation temperature for determination of results. Glucose is fermentatively utilized, with the formation of acid; gas and hydrogen sulfide are not produced. There is no growth in the presence of KCN. Phenylalanine and tryptophan are not deaminated, gelatin is not liquified, lysine is not decarboxylated, and arginine is not dehydrolyzed. Nitrate is reduced to nitrite, catalase is produced, but oxidase is absent. Acetoin is produced at 25 to 28°C (not at 37°C) by most strains of *Y. enterocolitica*, *Y. frederiksenii*, *Y. intermedia*, and *Y. aldovae*, but not by the remaining *Yersinia* species. With the exception of *Y. pestis*, urease is produced at 25-28°C. Ornithine is decarboxylated by all the species except *Y. pestis* and *Y. pseudotuberculosis*. In almost all cases, positive identification of *Y. pestis* is dependent upon recognition of at least one of the unique determinants listed:

	Indole	Omithine	Motility 25-28°C	Sucrose	Sorbitol
<i>Y. pestis</i>	-	-	-	-	-
<i>Y. pseudotuberculosis</i>	-	-	+	-	-
<i>Y. enterocolitica</i>	V (50)	+	+	+	+
<i>Y. frederiksenii</i>	+	+	+	+	+
<i>Y. intermedia</i>	+	+	+	+	+
<i>Y. kristensenii</i>	V (50)	+	+	-	+
<i>Y. aldovae</i>	-	+	+	V(20)	+

**Lysis by bacteriophage:** Lysis by the *Y. pestis* bacteriophage ΦA1122 is used to conclusively identify *Y. pestis* and differentiate it from *Y. pseudotuberculosis*. At 35°C, ΦA1122 phage is lytic to both species, but at 25°C it only causes lysis of *Y. pestis*. The test utilized typically uses ΦA1122 impregnated filter paper strip as the test reagent (20, 52).

Animal testing can be used if heavily contaminated specimens must be examined. Fleas are usually examined by this method. The samples are homogenized in sterile saline, of which 0.1 to 0.2 ml are subcutaneously or intraperitoneally inoculated into mice, rats, or guinea pigs. If virulent *Y. pestis* organisms are present, the animals die within 2 to 6 days and the organisms can be isolated from the blood, liver, and spleen.

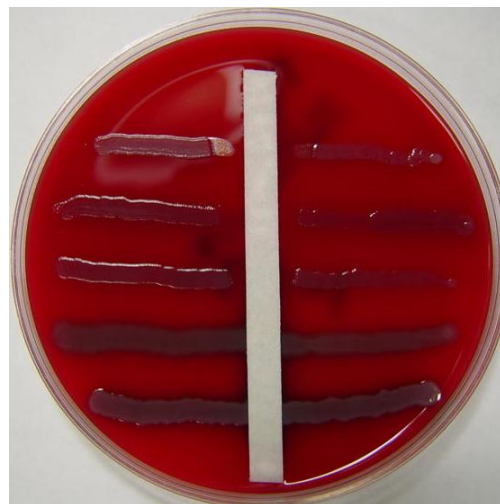


Figure 24. *Y. pestis* (top) are sensitive to A1122 phage at both 25°C and 35°C whereas other *Yersiniae* are not (bottom 2).

### Commercial Identification systems.

NOTE: See Appendix E for technical descriptions of the methods.

NOTE: As *Y. pestis* is relatively nonreactive, commercial tests may identify it as *Shigella* species, H<sub>2</sub>S-negative *Salmonella*, *Acinetobacter* species, or *Y. pseudotuberculosis* [Note: THIS IS EXTREMELY IMPORTANT TO CONSIDER]. *Yersinia* spp. is included in the databases of most commercial identification systems, such as Microscan, Vitek, API, Biolog, and BBL Crystal ID (4, 70). In 60% of human plague cases, it was the identification of an organism as *Y. pestis* by one of these systems that first led to a diagnosis of human plague. Not all differential tests for *Yersinia* spp. are included in these identification systems, however, which are furthermore based on results obtained at 35°C. Slow growth of field strains, especially of *Y. pestis*, in artificial media, and a low level of biochemical activity at 35°C, may lead to wrong or doubtful identifications, especially in the hands of inexperienced laboratory workers. Although most isolates may be correctly identified to the genus level, it is strongly advised to confirm commercial identification systems.

Fatty acid methyl ester (FAME) analysis: All *Y. pestis* strains displayed some major fatty acids, namely, the 12:0, 14:0, 3-OH-14:0, 16:0, 16:1 omega-9-cis, 17:0-cyc, and 18:1 omega-9-trans compounds. While these are analogous to fatty acids in other *Yersinia* species, the fatty acid compositions could be separated into three clusters corresponding to (i) nonpathogenic strains and species of *Yersinia*, (ii) pathogenic *Y. enterocolitica* isolates, and (iii) *Y. pseudotuberculosis* and *Y. pestis* strains. A dangerous pathogens database is available as a separate library. The determination of *Y. pestis* is included in the library. The system does a good job of resolving *Y. pestis* from the other *Yersinia* spp. but some misidentification occurs (34).

Vitek: The previous Vitek (a legacy system with 32 chemical reactions) had *Y. pestis* in the database but often misidentified *Y. pestis* as *Y. pseudotuberculosis*. The Vitek 2 using the GNI card will do a fairly good job of identifying *Y. pestis*. The Vitek GNI card was used to identify 212 isolates of 10 *Yersinia* species. Identification was correct for 96.3% of the isolates (156 of 162) to the genus level and for 57.4% of the isolates (93 of 162) to the species level for *Yersinia* spp. listed in the Vitek database. We recommend additional identification methods for isolates assigned to the genus *Yersinia* by the Vitek system (35).

Biolog: While the GN2 system had a Dangerous Pathogens database for identification of *Y. pestis*, the current GENIII does not. *Y. pestis* identification by Biolog has not been well studied and few data are available on the accuracy of the identification done by the GENIII system.

### Immunoassay Tests.

NOTE: For a short explanation of the principles of the assay technologies, see Appendix C.

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

#### Direct agent immunoassays.

Lateral flow immunochromatography: Hand-held assays (HHA), "Smart tickets", or lateral flow assays are commercially available and provide potentially useful. Several lateral flow or hand-held assays for *Y. pestis* have been developed. Most use the F1 capsule as the target antigen. Assays have a reported sensitivity of 0.5 ng F1/ml and they have been used to confirm suspected plague in Madagascar (14, 54, 65, 72).

Direct fluorescent antibody (DFA): A positive DFA for the F1 antigen can be used as presumptive evidence of a *Y. pestis* infection. The F1 antigen is predominantly expressed by *Y. pestis* at 37°C. Samples that have been refrigerated for more than 30 h, cultures that were incubated at room temperatures less than 35°C, or samples from fleas, will be negative (44).

Enzyme-linked immunosorbent assay (ELISA): ELISAs targeting the F1/capsule antigen has been developed. Sensitivity of F1 ELISAs have been reported to be 100% in bubo aspirates, 52% in serum, and 58% in urine specimens. In culture-negative patients, the F1 ELISA was positive in 10% of bubo aspirates, 5% of serum, and 7% of urine specimens from those that seroconverted (13, 40, 62).

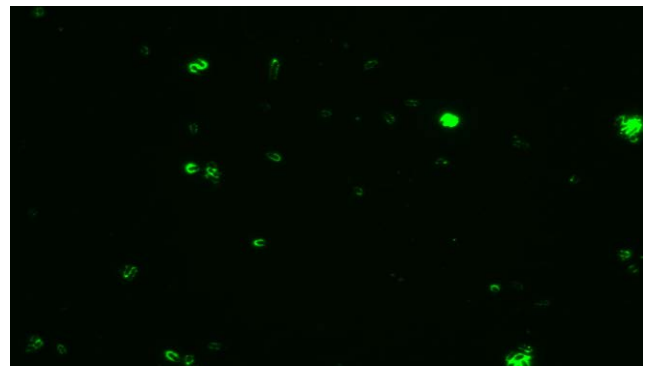


Figure 25. Direct fluorescent antibody test for *Y. pestis* demonstrated they typical apple-green fluorescence of a positive assay.



**Bioluminescence - AB cell-based sensor:** A pathogen sensor that uses B lymphocytes has been developed. B cell lines were engineered to express cytosolic aequorin (a calcium-sensitive bioluminescent protein from the *Aequoria victoria* (jellyfish) as well as membrane-bound antibodies specific for pathogens of interest. When antibodies crosslink, elevated intracellular calcium concentrations cause the aequorin to emit light. The sensor is named CANARY (Cellular Analysis and Notification of Antigen Risks and Yields). Cells specific for *Y. pestis*, could detect as few as 50 colony-forming units (CFU) in a total assay time of less than 3 min. The probability of detection for *Y. pestis* ranged from 62% for 20 CFU to 99% for 200 CFU, whereas the false-positive rate for the CANARY assay was 0.4%. Cells did not respond to large numbers of unrelated bacteria. The system has low stability and needs a constant replenishment of the B-cells however (56).

**Serological testing.** The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

**Enzyme-linked immunosorbent assay (ELISA):** For the detection of *Y. pestis*, the capsular F1 antigen is the antigen typically used. Typically, a titer of > 1:10, or a fourfold rise or fall of the antibody titer, is confirmatory of recent infection in persons who have not previously been infected or vaccinated. (43).

**Passive hemagglutination (HA):** For HA, the use of highly purified F1/capsule antigen is essential for good specificity. The passive HA test is sensitive and constitutes an effective means of detecting recovery from plague. Heat inactivated sera (56°C for 30 mins) with 1:100 with normal rabbit sera is used. Again, the criteria of a titer of > 1:10, or a fourfold rise or fall of the antibody titer, is confirmatory of recent infection in patients who have not previously been infected or vaccinated (15).

**Complement fixation test (CFT or CF):** The CFT is not as sensitive in clinical diagnostics as is ELISA or HA, but is good for determining the antibody content of sera from immunization studies. Like HA, purified F1/capsule antigen, titrations of hemolysin, complement, and complement controls are needed prior to testing (15).

**Immunomagnetic Separation - Flow cytometry detection method:** A combination of immunomagnetic separation and flow cytometry for the serodiagnosis of human plague has been reported. Paramagnetic beads are indirectly coated with F1 capture antigen. The assay showed the same sensitivity as the ELISA and almost the same specificity (99.0 versus 100%) as the immunoblot (61).

## Nucleic Acid Detection Tests.

There have been a number of polymerase chain reaction (PCR) assays developed for *Y. pestis* and most are adequate for general use (29, 31, 36, 50). For routine purposes, primers to the virulence plasmids are typically sufficient to make a presumptive identification. Primers to the *pgm* loci can also be used to study virulence of the strains, but several primer sets are needed to adequately cover the *pgm* loci and determine its absence, versus select mutations or rearrangements in the loci (10, 25, 27, 39, 41, 66). The most commonly used target for *Y. pestis* diagnostic assays is the F1 capsule (*pMT/pFra*) gene (45). Other targets for molecular detection include the pesticin (*pst*), plasminogen activator (*pla*) or pesticin immunity protein (*pim*) genes on the *pPCP/pPst* plasmid.

*pla*  
*pst*  
*caf1*  
 LCR (*lcrR*, *lcrG* or *lcrV*)  
*yops* (*yopE*, *yopM*)

As these plasmids and genes can potentially exist in non-*pestis* strains, combinations of two or more assays are probably needed. Conventional operational procedure is that two separate biomarkers are needed for a presumptive positive call, therefore the two targets should be on separate plasmids and not on the same plasmid. Targeting both *pim* and *pla* (both on *pPCP1*), does not ensure independence of the assay results. Plasmids are known to be promiscuous with transfer among organisms, resulting in the potential for a false-positive. Targeting two independent plasmids increases the probability that a false-positive will be recognized. Although *pla* is purported to be specific for *Y. pestis*, it has a close analog of *E. coli* *OmpT*, an outer membrane protein shared by many enteric bacteria that could cause false-positive reactions. The use of *pPCP1* only, as a target, is highly discouraged.

Amplification of a 23S rRNA sequence specific to *Y. pestis*. The identification of sequences of *Y. pestis*-specific *rpoB* ( $\beta$ -subunit of RNA polymerase) provides another amplification target area.

**Chromosomal DNA Amplification:** A primer pair that anneals to a specific *Y. pestis* chromosomal DNA sequences has been reported. The primers amplified a 276 bp product from strains of the three recognized biotypes (biovars) of *Y. pestis*. The primers did not amplify sequences from *Y. pseudotuberculosis* and did not cross-react with a collection of DNAs from bacterial, viral, and mammalian sources (53).

**Antibiotic-resistance PCR:** An assay to detect ciprofloxacin resistance (Cip(r)) in *Y. pestis* has been developed with two groups of fluorogenic probes. The first group included a probe homologous to the wild type *Y. pestis* gyrA sequence with two corresponding probes that were homologous with two different mis-sense mutations in codon 81 of GyrA. The second group of probes included a wild type probe and two corresponding probes that recognized mis-sense mutations in codon 83 or gyrA. Cip(r) was found to be due to one of four point mutations in gyrA that altered codon 81 or 83 (37).

**The Joint Biological Agent Identification and Diagnostic System (JBAIDS):** The JBAIDS Plague Detection Kit is a real-time polymerase chain reaction (PCR) test kit intended for the qualitative *in vitro* diagnostic (IVD) detection of target DNA sequences of *Y. pestis*. The kit can be used to test human whole blood collected in sodium citrate or sputum collected aseptically from individuals greater than 18 years of age suspected of having septic or pneumonic plague. In addition, positive blood cultures and colonies may be tested. The JBAIDS Plague Target 2 assay is used as a supplementary test only after a positive result with the Target 1 Assay. The JBAIDS Plague Target 1 and Target 2 assays are run on the JBAIDS instrument using the Diagnostic Wizard. Results are for the presumptive identification of *Y. pestis* in conjunction with culture and other laboratory tests. The definitive identification of *Y. pestis* from colony growth, liquid blood culture growth, or from blood or sputum specimens requires additional testing and confirmation procedures in consultation with public health or other authorities for whom reports are required. The diagnosis of plague must be made based on history, signs, symptoms, exposure likelihood, and other laboratory evidence in addition to the identification of *Y. pestis* from cultures or directly from whole blood or sputum specimens.

## Other Diagnostic Methods.

None identified.

## Characterization.

(see Appendix C. Diagnostic Technologies for a method descriptions)

Small genetic differences in *Y. pestis* need to be identified for characterization to strain level. Specific detection techniques (e.g., MLVA-multilocus variable tandem repeat analysis, IS100 region genomic analysis, and genomic-synonymous SNP analyses) allow the identification of individual *Y. pestis* variants from detailed phylogenetic analyses (2, 3, 33). While the new techniques add new tools for scientific study, (38) there are complexities and limitations of applying molecular techniques to elucidate the ecological constraints placed on pathogens. For example, the independent replication of selected (e.g., IS100) genetic units may represent selection independence from the plague organism itself. Although promising for mapping strain evolution quantitatively, these techniques also do not reveal potentially miniscule qualitative differences that may be selected strongly in response to underlying ecological forces (21, 33).

**Traditional Ribotyping:** The rRNA gene restriction fragment length polymorphisms of 70 strains of *Y. pestis* were determined by hybridization with a 16S-23S rRNA probe from *Escherichia coli*. The combination of *EcoRI* and *EcoRV* patterns resulted in the elucidation of 16 distinct ribotypes. The *EcoRI* rRNA gene restriction patterns of *Y. pestis* and *Y. pseudotuberculosis* differed greatly. In one of the first studies on molecular characterization of the species, Guiry et al. identified 20 patterns (A through T) within the three biovars based on ribotyping. The repertoire of ribotypes corresponded, to a degree, with the biovars Orientalis, Antique, and Medievalis (17, 22-24, 30, 32, 67).

**VNTR Analysis:** A tetranucleotide repeat sequence, CAAA, was identified in the genome of *Y. pestis* that can be used for variable-number tandem repeat (VNTR) analysis. The repeat is in an intergenic region between two tentatively identified open reading frames. VNTR analysis has a greater discriminatory capacity than the ribotyping method; however, VNTR analysis examines a more limited region of the genome than pulsed-field gel electrophoresis (3, 30).

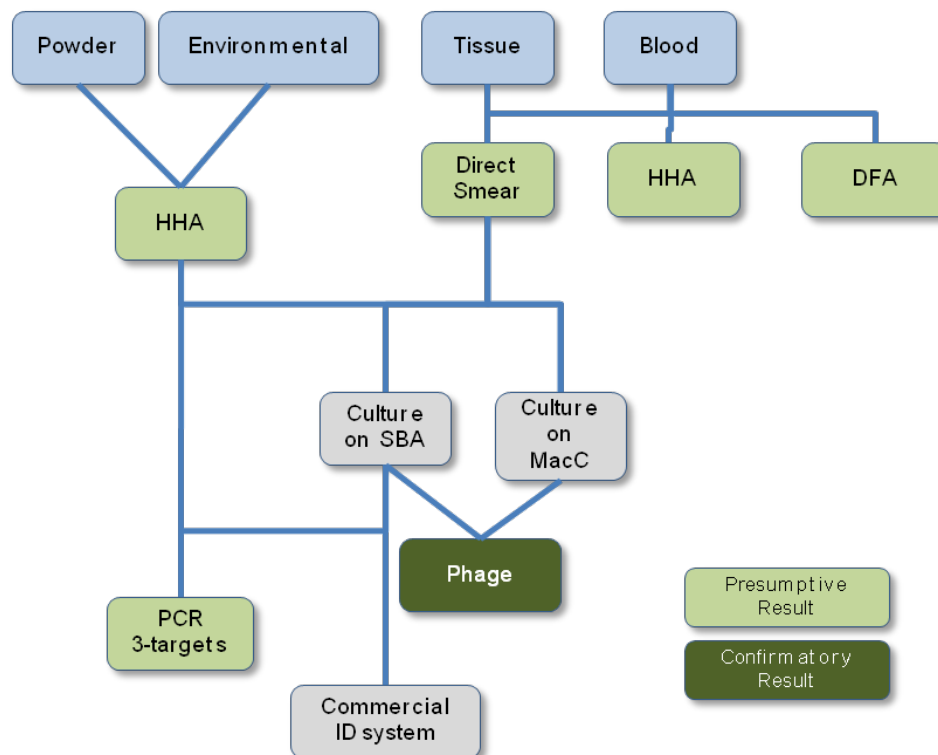
**PFGE Analysis:** Pulsed-field gel electrophoresis (PFGE) analysis is used to perform epidemiological investigation of the genetic variability of *Y. pestis* in the United States. This technique provides an increased ability to discriminate between strains and therefore can significantly improve epidemiological studies related to the origin of new plague isolates (30). PFGE, however, is more labor intensive and takes longer to complete.



## Confirmation.

Presumptive identification of a biological threat agent is achieved by the detection of a biological marker using a single test methodology (for example, hand-held assay (HHA)). Presumptive diagnosis can also be made by Gram, Wright, Giemsa or Wayson stain of blood, sputum, cerebrospinal fluid, or lymph node aspirates.

Civilian definitive identification or confirmation of *Y. pestis* requires culture of the organism with positive results from the A1122 phage assay. Culture of the organism precludes false-positive results from non-specific interference and allows the evaluation of false-negative results in the context of the broad array of testing performed. Definitive identification or confirmation testing is performed at sanctioned reference laboratories, including reference laboratories of the CDC Laboratory Response Network (LRN). Specific LRN protocols and reagents are proprietary but any definitive identification or confirmation typically follows a general scheme:



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# *Francisella tularensis*

## OVERVIEW

*Francisella tularensis*, the causative agent of tularemia, is a small, aerobic non-motile, gram-negative coccobacillus. Tularemia (also known as rabbit fever and deer fly fever) is a zoonotic disease that humans typically acquire after skin or mucous membrane contact with tissues or body fluids of infected animals, or from bites of infected ticks, deerflies, or mosquitoes. Less commonly, inhaling contaminated aerosols or ingesting contaminated foods or water may produce clinical disease. Respiratory exposure to infectious aerosols would typically cause typhoidal tularemia with pneumonia, but rarely ulceroglandular or oculoglandular forms can be seen as well. The organism is found throughout the temperate northern hemisphere and is typically the cause of only sporadic human disease (average of 124 cases per year in the U.S. from 1990-2000), but is infrequently the cause of large human epidemics associated with animal epizootics. *F. tularensis* exists in at least two virulent species: Type A and Type B. The organism can remain viable for weeks in water, soil, carcasses, hides, and for years in frozen rabbit meat. It is resistant for months to temperatures of freezing and below. It is easily killed by heat and disinfectants.

*F. tularensis* subspecies *tularensis* (type A) and *F. tularensis* subspecies *holarctica* (type B) are the two most virulent strains of this expanding group of organisms. Until recently, *F. tularensis* type A or B were restricted to the northern hemisphere, where *F. tularensis* type A or B are common in North America but only *F. tularensis* type B is typically found in Europe and Asia. *F. tularensis* is relatively easy to grow and growth is required for confirmation, typically by the direct fluorescent antibody assay.

## ORGANISM INFORMATION

### Taxonomy Information.

Genus *Francisella*. In the last 3-4 years, there has been an increase in the officially recognized species and subspecies within the Family *Francisellaceae*. There are now **six** recognized species of *Francisella* and it is expected to continue to grow, as more species are isolated and characterized (79).

- a. *F. tularensis* – the classical *Francisella* species from 1912
  - b. *F. philomiragia* – recognized as a separate species in 1969; *F. philomiragia* appears not to be highly virulent and is mostly an opportunistic agent, infecting patients with a underlying condition that renders them more susceptible. Between 1974 and 1997, only 15 cases have been described in the literature. All but one involved a host with an impaired physical barrier to infection (near- drowning) or impaired immunologic defenses (chronic granulomatous disease or myeloproliferative disease) (23, 33). The drowning and water exposure cases were associated with saltwater, in contrast to *F. tularensis* infections, which are associated with freshwater sources (37).
  - c. *F. hispaniensis* – a newly recognized separate species in 2010 was isolated from human blood in 2003. Cysteine is not required but enhances growth (34).
  - d. *F. noatunensis* – recognized as a separate species in 2007 and incorporated the provisional *F. piscicida* species. These are the ‘fish’ *F.* occurring in wild and farmed fish. There are two distinct subspecies and a proposed third subspecies. They are considered psychrotrophic (cold loving; 10-25°C) organisms. Cysteine is required for growth (28, 54, 59). Two subspecies have been added to the taxonomy, *F. noatunensis* subsp. *noatunensis* in 2007 and *F. noatunensis* subsp. *orientalis* in 2009 (60).
  - e. *F. guangzhouensis*- first published as a separate species in 2013, this is a ‘new’ *Francisella* isolated from water of air-conditioning systems of various cooling towers in Guangzhou city, China (68, 69).
  - f. *F. halioticida*, - first published as a separate species in 2011, this is another ‘fish’ *Francisella*, isolated from diseased abalone in Japan (6). Placement into a separate species was based on phenotypic and sequence difference (in 16S and nine housekeeping genes).
2. *F. tularensis* subspecies:



- a. *F. tularensis* subspecies *tularensis* (type A; also referred to as subspecies *nearctica* by investigators in the former Soviet Union) is highly infectious and virulent; it is found primarily in North America and two distinct genetic clades with two distinct minor clades have been identified (44, 45).
    - (1) A1: A1a and A1b; human infections due to A1b resulted in significantly higher mortality (24%) than those caused by A1a (4%)(66). New terminology for the the A1 group is based on whole-genome analysis and the assignment of subgroups based on single nucleotide polymorphism analysis (5).
    - (2) A2: A2a and A2b; human infections due to A2 were not fatal (0%).
      - (a) Cats and lagomorphs are reported as primary sources of type A infections in humans.
      - (b) Type A infections have been strongly linked to tick transmission with A1 transmission correlating with both *A. americanum* and *D. variabilis*, whereas the A2 subpopulation is linked only to the tick *D. andersoni*. Deerfly or horse-fly transmission may also occur (65).
  - b. *F. tularensis* subspecies *holarctica* (type B; formerly referred to as *palaeartica* or *paleartica* in some sources): less virulent (7%) compared to Type A; three biovars have been identified:
    - (1) Biovar I: erythromycin sensitive; primarily found in North America, Europe, Siberia, the Far East, and Kazakhstan. In Europe, this group probably corresponds to group B.FTNF002-00, but erythromycin resistant was not addressed in a study of eastern versus western European *Francisella* strains (32). In the US, either B.Br.OSU18 or B.Br.OR96-0246 types predominate (90).
    - (2) Biovar II: erythromycin resistant; primarily found in Western Europe. This group probably corresponds to group B.13 but erythromycin resistant was not addressed in a study of eastern versus western European *Francisella* strains (32).
    - (3) Biovar japonica: found predominately in Japan.
      - (a) Rodents (including beavers, voles, prairie dogs, and squirrels) and primates are the most commonly reported sources for human infections (62). Type B is more typically associated with waterborne outbreaks, especially in northern and eastern Europe.
      - (b) Type B arthropod transmission is strongly linked to mosquitos and has only been identified in the tick *D. variabilis* in the northern states of Montana and South Dakota. Deerfly or horse-fly transmission may also occur (65).
  - c. *F. tularensis* subspecies *mediasiatica*: found in the Central Asian republics of the former Soviet Union. Virulence is similar to *F. tularensis* subspecies *holarctica*.
  - d. *F. tularensis* subspecies *novicida*: considered to be of low virulence and generally causes illness only in immunocompromised hosts
3. *F. tularensis* and *F. novicida* traditionally have been considered separate species; however, the current approach is to consider *F. novicida* as a subspecies of *F. tularensis* - *F. tularensis* subsp. *novicida*. Until 1989, a single 1951 Utah water isolate was defined as *F. tularensis* subsp. *novicida* and no human infection had been linked with it. During a subsequent study of 16 unusual human bacterial isolates, 14 were identified as *F. philomiragia* and two were identified as *F. tularensis* subsp. *novicida*. The two latter isolates, along with two from Texas, are the only ones causing human cases described in the literature. A human isolate was recovered from a cervical lymph node of a patient in Utah in 2000 (2, 17, 20, 21, 47). *F. novicida* is uniquely different from the other *F. tularensis* strains that it should be a separate species. The CDC has exempted *F. novicida* from select agent (BSAT) controls based on the the lack of human virulence so far, and the molecular characterization demonstrating it as a unique organism with less than 60% genomic similarity.

## Virulence Factors.

Virulence factors for *F. tularensis* have not been especially well characterized. Like other gram negative, intracellular bacteria, a number of factors have been proposed as virulence factors, but there does not seem to be any specifically novel *F. tularensis* virulence factors. In addition, the virulence factors proposed were identified by differences in virulence between a fully functional system and a mutated

system. That premise makes an indirect association between a mutation and a loss of virulence, not a direct one (the mutation may be in an accessory system that feeds or regulates another factor that is specifically the virulence factor).

Different hemolytic agents, which may facilitate degradation of the phagosome, include NlyA and acid phosphatase AcpA. Both of these virulence factors were a result of work on *F. novicida* and not on the human pathogen *F. tularensis* (11, 46).

The ATP binding cassette (ABC) proteins may be linked to the secretion of virulence factors. Another protein is a homologue to a disulphide oxidoreductase, DsbA, an outer membrane lipoprotein in *Francisella*. A *dsbA* mutant was found to be severely attenuated for virulence (86).

*F. tularensis* has a pathogenicity island (FPI) that includes genes for several proteins linked to virulence (7, 12, 57, 73). One protein, IgIC (23-kD protein), is required for phagosomal breakout and intracellular replication. The absence of this protein allows *F. tularensis* to die and be degraded by the macrophage. Transposon mutagenesis mutations in nine genes led to marked attenuation with an LD<sub>50</sub> of >10<sup>3</sup> CFU: *rplA*, *wbtI*, *iglB*, *iglD*, *purL*, *purF*, *ggt*, *kdtA*, and *glpX*. Several of these genes are on the FPI. *F. tularensis* uses type IV pili to bind to host cells and facilitate phagocytosis. Mutant strains lacking this pili show attenuated pathogenicity. The type IV secretion system and pili are on the FPI.

## Epidemiology and Endemic Areas

Several thousand cases annually were reported in the United States until the 1940s, when the incidence of reported cases started a steady decline. All states, except Hawaii, have reported sporadic cases. Most cases, however, occur in south-central and western states. The gross epidemiology of tularemia in the United States has not changed significantly in the past several decades, but new insights into strain level epidemiology is evolving (56, 66). It remains a rural disease, and most patients acquire tularemia from tick or deerfly bites, or by contact with infected animals, particularly rabbits.

*F. tularensis* subsp. *tularensis* was thought to occur only in North America, where it has been most closely associated with infection in lagomorphs and humans. Recently it was found that *F. tularensis* subsp. *tularensis* appears not to be limited to North America as it has been isolated from mites and fleas in Europe (30). In contrast, infection with *F. tularensis* subsp. *holarctica* is less virulent; it has a much wider distribution, in North America, Europe, and Asia. Type B is associated with a greater variety of animals, primarily hares and rodents, and with contaminated environmental source outbreaks. Until recently, the occurrence of *Francisella* has been limited to the northern hemisphere, principally in North America, Europe, and the northern section of Asia. Recently, two isolates have occurred in the Southern Hemisphere of interest. *F. tularensis* subsp. *novicida* has been described primarily in North America (4, 9, 33). In 2003 the isolation and identification of a *novicida*-like subspecies of *F. tularensis* from a foot wound sustained in brackish water in the Northern Territory of Australia was reported (94). In 2008 *Francisella novicida* was isolated from a woman in Thailand who was receiving chemotherapy for ovarian cancer (49). While initially reported as *F. tularensis* subsp. *novicida*, evidence exists that these two isolates actually represent a new and unnamed species. In 2012 an isolate of *F. tularensis* subsp. *holarctica* was reported a woman bitten by a ringtail possum in Tasmania, Australia. This case is the first incident of *F. tularensis* type B in the Southern Hemisphere (35). *F. tularensis* subsp. *mediasiatica* is found in regions in Kazakhstan and Turkmenistan and has been isolated from hares and ticks but not from humans (72, 78, 90). Of the 15 known isolates of *F. philomiragia*, one was recovered from Switzerland and the rest were recovered in North America (Hollis, 1989 #3034, 93).

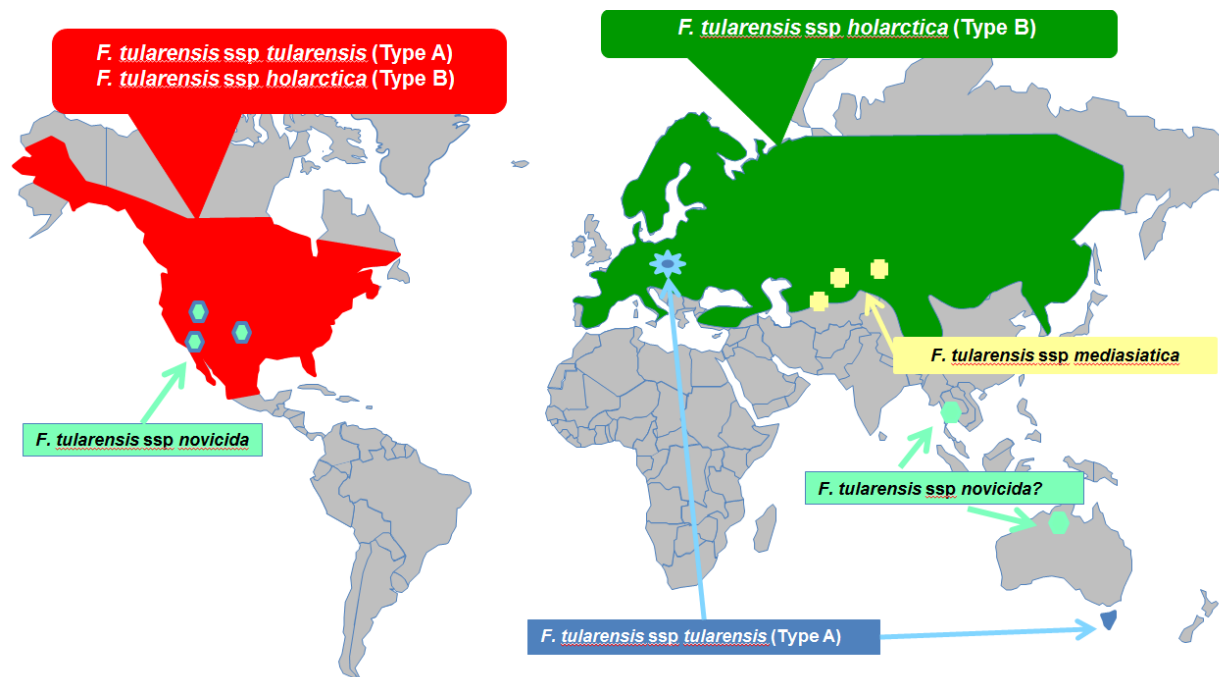
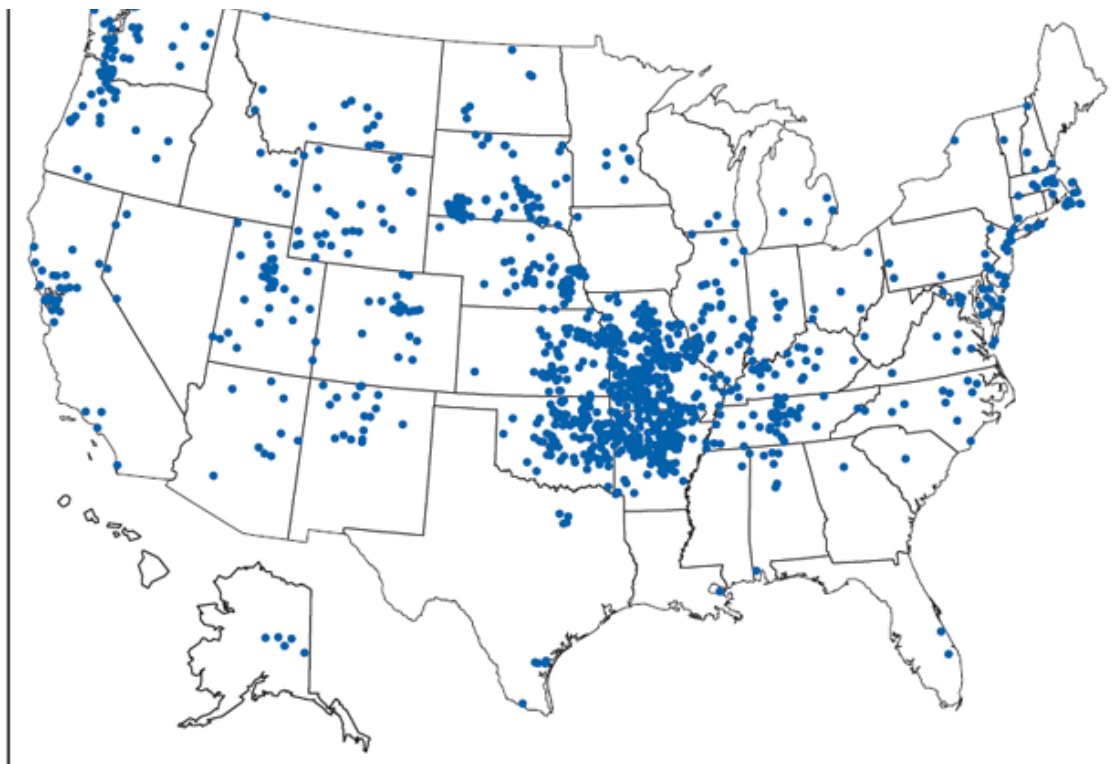
Figure 26. World-wide distribution of *F. tularensis*

Figure 27. In the US there are an average of 124 cases per year. Four states account for ~60% of all reported cases (OK, AR, MO, SD) Morbidity and Mortality Weekly Report (MMWR) November 29, 2013 / 62(47);963-966.

## LABORATORY DIAGNOSTICS

### Biosafety Information.

Biosafety in Microbiological and Biomedical Laboratories (BMBL) Containment Recommendations (5th Edition): Biosafety level (BSL)-2 practices, containment equipment, and facilities are recommended for activities involving clinical materials of human or animal origin suspected or known

to contain *F. tularensis*. Laboratory personnel should be informed of the possibility of tularemia as a differential diagnosis when samples are submitted for diagnostic tests. BSL-3 and animal BSL-3 practices, containment equipment, and facilities are recommended for all manipulations of suspect cultures, animal necropsies and for experimental animal studies. Preparatory work on cultures or contaminated materials for automated identification systems should be performed at BSL-3. Characterized strains of reduced virulence such as *F. tularensis* Type B (strain LVS) and *F. tularensis* subsp *novicida* can be manipulated in BSL-2 (Note: In 2014 all *F. tularensis* subsp *novicida* were made exempt agents. Previously, only the *F. tularensis* subsp *novicida* strain U112 was considered an exempt strain). Manipulation of reduced virulence strains at high concentrations should be conducted using BSL-3 practices.

## Diagnostic Information

### General.

*F. tularensis* is a gram-negative, non-motile, encapsulated, non-spore forming, coccobacillary or rod-shaped, strict aerobe, with a width of 0.2 µm and a length of 0.2–0.7 µm. It causes the disease called “tularemia” and is principally a zoonotic disease with human infections being incidental.

### Staining

Gram stain: Direct examination of bacterial micromorphology may demonstrate small, pale-staining, gram-negative coccobacillus. Gram staining on direct samples offers no diagnostic utility as the results is very non-descriptive.

## Metabolic Information:

*F. tularensis* is an obligate aerobic but growth is stimulated by increased CO<sub>2</sub> and may require 2 to 5 days before colonies are visible on agar medium. The organism has commonly been reported to have a growth requirement for the amino acids cysteine and cystine. The cysteine and cystine requirement is more pronounced for laboratory passed strains than on fresh cultures. On initial culture, specimens such as blood or tissues provide an intrinsic source of sulfhydryl compounds that permit *F. tularensis* growth. Upon subculture, the fastidiousness of *F. tularensis* becomes evident as the exogenous compounds are depleted, leading to the loss of its viability unless the subculture is propagated on a cysteine and cystine-supplemented medium.

Strains of *F. tularensis* ssp tularensis produce acid in media containing either glucose or glycerol as a carbon source.

Optimal temperature: 35-37°C.

Upper temperature:

- i) *F. tularensis* subspecies *tularensis* typically light growth at 42°C
- ii) *F. tularensis* subspecies *holarctica* 42°C
- iii) *F. tularensis* subspecies *novicida* 50°C
- iv) *F. tularensis* subspecies *mediasiatica* unkn
- v) *F. philomiragia* – light to no growth at 50°C

Lower temperature:

- i) *F. tularensis* subspecies *tularensis* no to light growth at 25°C
- ii) *F. tularensis* subspecies *holarctica* typically light growth at 25°C
- iii) *F. tularensis* subspecies *novicida* grows at 25°C
- iv) *F. tularensis* subspecies *mediasiatica* unkn
- v) *F. philomiragia* grows at 25°C

Optimal pH: not stated

### General Culturing Information

Sheep blood agar (SBA): The organism does not grow well, but usually does grow in 48 hrs, on commercial sheep blood agar. Initial isolation from blood or tissue grows better than do laboratory adapted strains. Laboratory strains are smaller than on media with cysteine or cystine. Colonies are non-hemolytic.



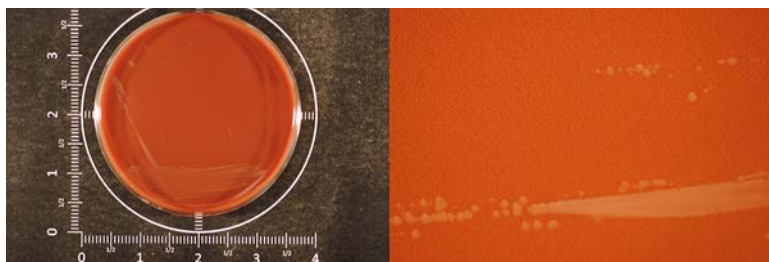
Figure 28. *F. tularensis* on SBA at 48hrs.

Chocolate agar (CA): Colony morphology of *F. tularensis* is most distinctive when it is grown on CA. *F. tularensis* colonies have an entire edge; they are about 2 mm in diameter after a 3-day incubation in a CO<sub>2</sub>, gray, smooth, raised, and moist with butyrous consistency. *F. tularensis* exhibits a prominent and unique opalescent sheen due to its production of H<sub>2</sub>S; this iridescent sheen is less prominent in *F. philomiragia* than in *F. tularensis*. Colonies of *F. philomiragia* on CA are >5 mm in size with an entire edge; they are white, smooth, raised, mucoid, and cysteine independent.



Figure 29 *F. tularensis* on chocolate agar at 48hrs.

Modified Thayer Martin (MTM): Modified Thayer Martin supports growth of all strains of *Francisella* spp. at 48 hrs. and is similar to growth on chocolate agar.



Glucose cysteine blood agar, Thayer-Martin agar [a Mueller-Hinton agar with 5% chocolate sheep blood and antibiotics]), Buffered charcoal-yeast extract agar: *F. tularensis* produces small, smooth, opaque colonies after 24 to 48 hr on medium containing cysteine or other sulfhydryl compounds.

	<i>F. tularensis</i> subspecies <i>tularensis</i>	<i>F. tularensis</i> subspecies <i>holarctica</i>	<i>F.</i> <i>tularensis</i> subspecies <i>novicida</i>	<i>F. tularensis</i> subspecies <i>mediasiatica</i>	<i>F. philomiragia</i>	<i>F. noatunensis</i>
<b>Chocolate Agar (CA)</b>	2-4 mm, raised, gray, smooth, moist, butyrous, entire			5 mm, raised, gray, smooth, moist, butyrous, entire	>5 mm, white, smooth, mucoid, entire	weak to no growth
<b>Glucose cysteine blood agar</b>	2 mm, pearl white to ivory with green tint and prominent opalescent sheen, smooth, butyrous, green-yellow discoloration of medium			5 mm, pearl white to ivory with green tint and prominent	>5 mm, creamy white to grey, mucoid, with	convex, pale white to grey colonies which reached

	opalescent sheen, smooth, butyrous, green-yellow discoloration of medium	purple tinted opalescent sheen,	maximum size (3-4 mm in diameter) in 48 h
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#### Selective Culturing Information

MacConkey agar- grows poorly (if at all)

Cysteine heart agar blood (CHAB) –antibiotic (CHAB-A): Improved recovery times and good sensitivity when cultures were inoculated on the site of an investigation using fresh tissues has been demonstrated. For contaminated specimens, antibiotic supplementation improved recovery by 81.1%. 7.5 mg of colistin, 2.5 mg of amphotericin, 0.5 mg of lincomycin, 4 mg of trimethoprim, and 10 mg of ampicillin per liter (64, 63). In internal laboratory testing, CHAB growth was suppressed and not as good as other media, (i.e.; chocolate or modified Thayer Martin) and required longer incubation (96hrs) to get good growth. But the use of CHAB for environmental isolation is recommended.



Figure 30. *F. tularensis* on CHAB at 96 hrs..

<b><i>Francisella</i> growth on various clinical media (percent of strains that grew)</b>				
	<i>Francisella tularensis</i>			<i>Francisella philomiragia</i>
	subsp. <i>tularensis</i>	subsp. <i>holartica</i>	subsp. <i>novicida</i>	
Chocolate Agar	100	100	100	100
Modified Thayer-Martin	100	100	100	100
Charcoal Yeast Dextrose Agar	100	100	100	100
Tryptic Soy Agar with 5% sheep blood	75	100	100	100
Thioglycollate Medium w/Dextrose, Hemin, Vitamin K	100	100	100	100
Brucella Agar	100	100	100	100
Brain Heart Infusion Agar	100	100	100	100
Cysteine Heart Agar with 9% Sheep Blood*	50	50	100	100
Tryptic Soy Agar	0	0	100	100
Thiosulfate Citrate Bile Salts Medium	0	0	100	100
Campy Blood Agar	0	0	100	0
Agar w/ 5% Sheep Blood (C-N-A)	0	0	0	0
MAC (MacConkey agar)	0	0	0	0



Sabourauds Dextrose Agar w/ Chloramphenicol, Cycloheximide	0	0	0	0
Schaedlers Medium w/Blood, Hemin, Vitamin K	0	0	0	0
Lowenstein-Jensen medium	0	0	0	0
Yersinia Selective Agar at 28°C	0	0	0	0
Yersinia Selective Agar at 35°C	0	0	0	0
• observational data disagreement with published literature				

## Diagnostic Tests:

The *Francisella* species are quite homogeneous, with only a few key differences serving to differentiate them. They all are oxidase-negative and weakly catalase-positive. Subspecies differentiation is less distinctive; single biochemical differences in glycerol fermentation and glucose utilization are used to define the biovars.

*F. tularensis* subspecies *tularensis* demonstrates citrulline ureidase activity (a difficult and cumbersome assay) and **most** utilize glycerol (which used to be the primary differentiation test between *tularensis* and *holarctica*).

*F. tularensis* subspecies *holarctica* does not demonstrate citrulline ureidase activity and three biovars have been identified:

- Biovar I: erythromycin sensitive.
- Biovar II: erythromycin resistant.
- Biovar japonica: found in Japan; citrulline ureidase negative.

*F. tularensis* subspecies *mediasiatica*.

*F. tularensis* subspecies *novicida*: *F. tularensis* subsp *novicida* can be distinguished from the other subspecies by its ability to grow independently of cysteine supplementation and by its comparatively larger vegetative cell size.

*F. philomiragia*: *F. philomiragia* is more biochemically reactive than *F. tularensis*; it also differs by its ability to ferment maltose and is oxidase positive using Kovac's reagent. They utilize glycerol and cannot utilize glucose.

Differentiation of Francisella from similar gram-negative genera								
Test	<i>F. tularensis</i>	<i>Brucella</i> spp.	<i>Bartonella</i>	<i>Acinetobacter</i> sp.	<i>Psychrobacter phenylpyruvicus</i>	<i>Oligella</i> sp.	<i>Borderella bronchiseptica</i>	<i>Hemophilus influenzae</i>
Oxidase	-	+	-	-	+	+	+	V
Urea hydrolysis	-	+	-	V	+	+	+	V
Gram stain morphology	Tiny ccb	Tiny ccb	Thin rod	Broad ccb	Broad ccb	Tiny ccb	Thin rod	Small ccb
X or V factor requirement	-	-	-	-	-	-	-	+
Cysteine enhancement	+	-	-	-	-	-	-	-
Motility	-	-	-	-	-	V	+	-
Major CFA	10:0; 14:0; 16:0; 18:1w9c; 3-OH-18:1; 0cyc	16:0; 18:1w7c; 18:0; 19; 0cyc	16:0; 17:0; 18:1w7c	16:1w7c; 16:0; 18:1w9c	16:1w7c; 16:0; 18:2; 18; 1w9c	16:0; 18; 1w7c	16:0; 18; 1w7c	14:0; 16; 1w7c; 16:0
+, greater than or equal to 90% positive: -, less than or equal to 10% positive: v, variable (11 to 89% positive): ccb, coccobacilli								

Test	<i>F. tularensis</i> subspecies <i>tularensis</i>	<i>F. tularensis</i> subspecies <i>holarctica</i>	<i>F. tularensis</i> subspecies <i>holarctica</i> - <i>japonica</i>	<i>F. tularensis</i> subspecies <i>novicida</i>	<i>F. tularensis</i> subspecies <i>mediasiatica</i>	<i>F. philomiragia</i>	<i>F. hispaniense</i>	<i>F. noatunensis</i>
Oxidase	--	--	--	--	--	+/+w	+	--
Motility	--	--	--/+w	--	--	--	unkn	--
H <sub>2</sub> S on TSI agar <sup>1</sup>	--	--	--	--	--	+w	-	--
Glucose (acid production)	+	+	+	+w	--	+w	unkn	+



Sucrose (acid production)	--	--	unkn	+w	unkn	+w	+(1)	+w
Glycerol (acid production)	+	--	+	+	+	unkn	+(1)	--
Maltose (acid production)	+	+	unkn	--	unkn	+w	-- (1)	+
Citrulline ureidase <sup>2</sup>	+	--	--	+	+	NA	unkn	unkn
1- <i>Francisella</i> spp. can produce H <sub>2</sub> S in cysteine supplemented media 2- Citrulline ureidase is currently a chromatographic method and not commonly performed, hence not very useful, and may not be accurate.								

### Commercial Identification systems

NOTE: See Appendix E for technical descriptions of the methods.

Fatty acid methyl ester (FAME) analysis: *Francisella* spp. have a unique cellular fatty acid composition and analysis of these strains revealed the presence of a large amount of 3-hydroxyoctadecanoate (3OH-18:O), that is not found in other bacteria. Consequently, these organisms may be identified readily by cellular fatty acid analysis using the Microbial Identification (MIDI) System (MIDI, Newark NJ) (15, 36).

Vitek: The original Vitek (32 chemical reactions) did not have *Francisella* in the database but the Vitek 2 does (10). Using the GN2 card, a positive call on the Vitek2 system is based on the positivity of 2 or 3 reactions out of 64, however. While the non-reactive nature of *Francisella* in the Vitek 2 GN2 card would seem to preclude highly reliable identifications, the Vitek 2 system does identify *Francisella* with accurate identifications in internal unpublished data with only 19 of 31 calls as "Nonreactive biopattern," "*Pseudomonas*," or "*Sphingomonas*" being the common misidentifications.

Biolog: While the GN2 system had a Dangerous Pathogens database for identification of *Francisella*, the current GENIII does not (51). Biolog recommends that fastidious organisms, like *F. tularensis*, *F. novicida* and *F. philomiragia* cultures be tested using their C2 protocol (inoculating fluid-C and inoculum density 62-68%). Because GENII software only contains *F. philomiragia* as a database entry, the other *Francisella* spp. were either mis-identified or provided 'no ID' (majority). Results from Biolog substrate use, however, were sub-species unique when analyzed separately from the Biolog system and potentially could be incorporated into an identification database.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer: MALDI-TOF was able to identify 45 *Francisella* to the species or sub-species level even when grown on different media (76).

### Immunoassay Tests.

NOTE: For a short explanation of the principles of the assay technologies, see Appendix C. Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

#### Direct agent immunoassays.

General. Immunoassays for the detection of *F. tularensis* are based on antibodies, polyclonal or monoclonal, against either *Francisella* outer membrane proteins (OMP) or the *Francisella* lipopolysaccharide (LPS) (26). Four outer membrane associated proteins are distinct to *Francisella* and provide good immunoassay sensitivity.

- b. ftu:FTT0583; fopA; outer membrane associated protein.
- c. ftf:FTF0583; fopA1; outer membrane associated protein.
- d. fth:FTH\_1293; fopA; outer membrane protein.
- e. ftn:FTN\_0756; fopA; OmpA family protein.

Lateral flow immunochromatography (LFI): Several entities have developed handheld assay (HHA), most targeting the 17-kD protein (TUL4) protein of *F. tularensis*. Compared to ELISA, the sensitivity of the HHA was about 100 times lower (29). In an evaluation of two commercial lateral flow assays, sensitivity was in the 10<sup>7</sup> to 10<sup>8</sup> "bacteria" per milliliter range therefore requiring retesting of negative samples by more sensitive methods (51, 96).

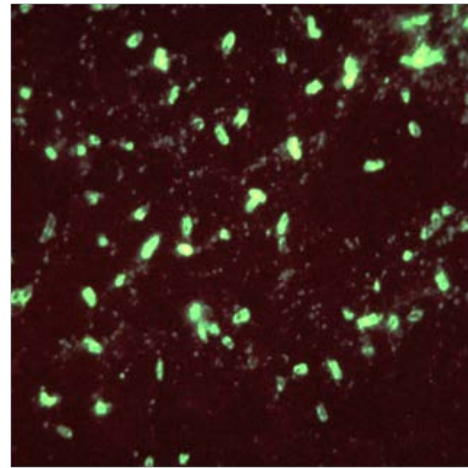
Slide agglutination: Slide agglutination assays have been reported as commercially available for direct testing of unknown cultures but seem to be based on the control reagents used to qualify the serological slide or tube assay. High titer polyclonal rabbit sera is typically used, but should be used with caution since the reagents have not been evaluated for this use. Polyclonal rabbit or a monoclonal anti-*F. tularensis* antibody have both been used (58).

Direct fluorescent antibody (DFA) assays: Some DFA reagents may have cross-reactivity with *F. philomiragia* and *Legionella* (43, 55, 70). DFA is the civilian confirmation method.

Time-resolved fluorescence (TRF) assay: *F. tularensis* immunoassay was converted to a TRF assays and tested the system in different matrices such as serum, urine, dirt, and sewage (61).

Enzyme-linked immunosorbent assay (ELISA): Several enzyme-linked immunosorbent assays (ELISA) have been developed that detect various *F. tularensis* OMPs or LPS. The typical sensitivity of ELISA assays are on the order of  $10^3$  bacteria/ml (29, 53, 89).

Electrochemiluminescence (ECL): An ECL assay for detecting *Francisella* cellular proteins has been developed for the legacy M1M system and provides sensitivity lower than that achieved by standard ELISA.



**Figure 31. Direct fluorescent antibody (DFA) for *Francisella tularensis* demonstrating classic apple-green fluorescence.**

Serological testing. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

Conventional serologic tests for *Francisella* exposure include the tube agglutination (TA) test, a microagglutination (MA) procedure, direct or indirect hemagglutination, or complement fixation tests. IgM antibodies may be detected as early as 2 weeks after infection, and can persist for more than 10 years. Since IgM antibody response can last for many years, its presence does not indicate acute infection (31, 77).

Tube agglutination (TA): The TA was one of the first methods used by the US Public Health Service to diagnose tularemia in patients (22).

Microagglutination (MA): The MA test was demonstrated more sensitive than the standard TA test for detecting anti-*F. tularensis* antibodies of the IgM class, 9 days earlier than the TA test (74). MA is essentially similar to TA except that safranin-stained tularensis antigen is used in round-bottom microtiter plates. Generally, a single specimen with a high titer of (>1:128 or 1:160) or a fourfold titer difference between acute- and convalescent-phase specimens taken at least 14 days apart, are considered positive. The formalin-killed whole-cell agglutination antigens have been reported to have cross-reactivity primarily with *Brucella*, *Proteus* OX19, and *Yersinia* organisms and care needs to be exercised in interpreting serology results (1, 8, 52).

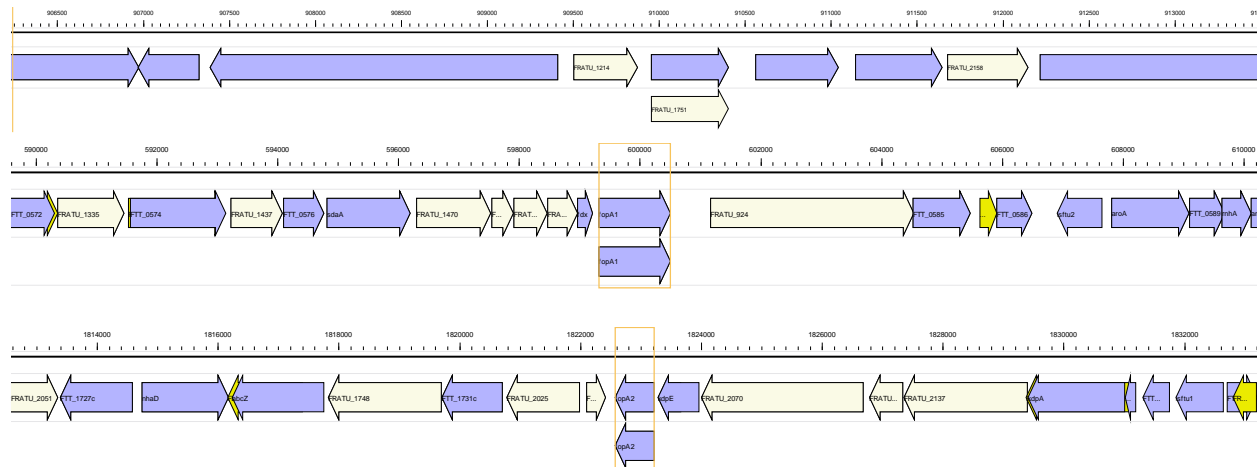
Enzyme-linked immunosorbent assay (ELISA): ELISA for detecting of tularemia-specific antibodies, using sonicated bacteria, purified outer membrane protein or lipopolysaccharide antigens, have been developed (3, 71, 75, 84, 89). Because cross-reactions can occur with *Brucella* spp. and because antibodies may persist for years after infection, diagnosis should be made only if a fourfold or greater increase is seen. Titers are usually negative the first week of infection, positive the second week in only 50-70% of cases, and reach a maximum titer in 4-8 weeks.

Indirect fluorescent antibody (IFA): An IFA *F. tularensis* IgG antibody assay is available that detects IgG class human antibody to *F. tularensis*, as an aid in the diagnosis of human infection by this pathogen (50).

### Nucleic Acid Detection Tests.

There have been a number of polymerase chain reactions (PCR) detection systems have been developed for *F. tularensis* and most are adequate for general use. For routine purposes, primers to one of the outer membrane protein genes provide a presumptive diagnosis (13, 14, 16, 21, 24, 25, 29, 38, 39, 41, 42, 80, 87, 88).

- Outer membrane lipoprotein - tul4 - a 17-kD lipoprotein
- Outer membrane lipoprotein - fopA - a 43-kD outer membrane protein
- Insertion element (ISFtu2) a 23kD gene that encodes a protein that is expressed upon macrophage infection
- OmpA-OmpF Porin (OOP) Family



**Figure 32. The tul4 (FRATU\_1751), fopA1 and fopA2 genes as located on *F. tularensis* Shu4 genome. These genes are the basis for many nucleic acid based assay targets.**

The Joint Biological Agent Identification and Diagnostic System (JBAIDS) Tularemia Detection Kit is a real-time polymerase chain reaction (PCR) test system intended for the qualitative in vitro diagnostic (IVD) detection of target DNA sequences of *F. tularensis*. The system can be used to test human whole blood collected in sodium citrate or sputum collected aseptically from individuals greater than 18 years of age suspected of having tularemia. In addition, positive blood cultures and colonies may be tested. This assay is intended to aid in the diagnosis of individuals presenting with signs and symptoms of pneumonic or typhoidal tularemia. It is not intended to aid in the diagnosis of glandular, ulceroglandular, oculoglandular, or oropharyngeal tularemia. The JBAIDS Tularemia Detection Kit is run on the JBAIDS instrument using the Diagnostic Wizard. Results are for the presumptive identification of *F. tularensis* in conjunction with culture and other laboratory tests. The definitive identification of *F. tularensis* from colony growth, liquid blood culture, blood specimens, or sputum specimens requires additional testing and confirmation procedures in consultation with public health or other authorities for whom reports are required. The diagnosis of tularemia infection must be made based on history, signs, symptoms, exposure likelihood, and other laboratory evidence, in addition to the identification of the target either from colonies, blood culture, whole blood specimens, or sputum specimens.

Single plasmids of various sizes have been associated with individual isolates of *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *novicida*, but are not currently PCR targets (67, 95). The functions of the plasmids are not well described and pretty much ignored.

### Other Diagnostic Methods.

None identified.

### Characterization.

(see Appendix C. Diagnostic Technologies for a method descriptions)

16s rRNA sequencing: Using 16s rRNA sequences, genus, species, and subspecies strains can be determined (20, 21, 72).

Other genes used for strain characterization include:

- rpoA*, B, D, H genes, (DNA-directed RNA polymerase subunit beta) differentiate between the other genera of *Francisella* but must be used with caution *Francisella* have been found to contain two *rpoA* genes which encode non-identical RNA polymerase (RNAP) subunits. The *rpoA* genes nevertheless are useful in phylogentic characterization.
- sdhA* (a putative succinate dehydrogenase) was found to differentiate strains at the subspecies level based on specific single-nucleotide polymorphism (SNP) signatures.
- recA* gene (recombinase A) gene that has been used in characterizing various strains of *Francisella*.
- fabH* gene (3-oxoacyl-[acyl-carrier-protein] synthase III gene) catalyzes the initial condensation and elongation reactions in fatty acid synthesis which in *Francisella*, are unique.

## DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

Repetitive PCR (repPCR, REP, ERIC or BOX): Rep-PCR and the similar other methods, use DNA primers corresponding to 35-150 bp repetitive elements. These methods have been applied to *Francisella* spp, but they were not discriminatory enough to distinguish individual strains and do not seem to be generally useful (39).

Pulsed-field gel electrophoresis (PFGE), PFGE with the restriction enzymes *XhoI* and *BamHI* revealed seven pulse-field types and allowed discrimination of the strains to the subspecies level. With *PmeI* enzyme, 4 distinct A genotypes, A1a, A1b, A2a, and A2b, and type B strains were discriminated (45, 81). Discrimination within the Type B strains was not significant, or the strains included were not representative of the Type B diversity.

Amplified fragment length polymorphism (AFLP) analysis produce a better degree of discrimination among *F. tularensis* subsp. *holarctica* strains (one primary cluster with three major sub-clusters and minor variations within sub-clusters) when *EcoRI*-C and *MseI*-A, *EcoRI*-T and *MseI*-T, *EcoRI*-A and *MseI*-C, and *EcoRI*-O and *MseI*-CA were used as primers. The degree of similarity among the strains was about 94%. The percent similarities of the AFLP profiles of this subspecies compared to those of *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *novicida*, and *F. philomiragia* were less than 90%, about 72%, and less than 24%, respectively, thus permitting easy differentiation of this subspecies (27).

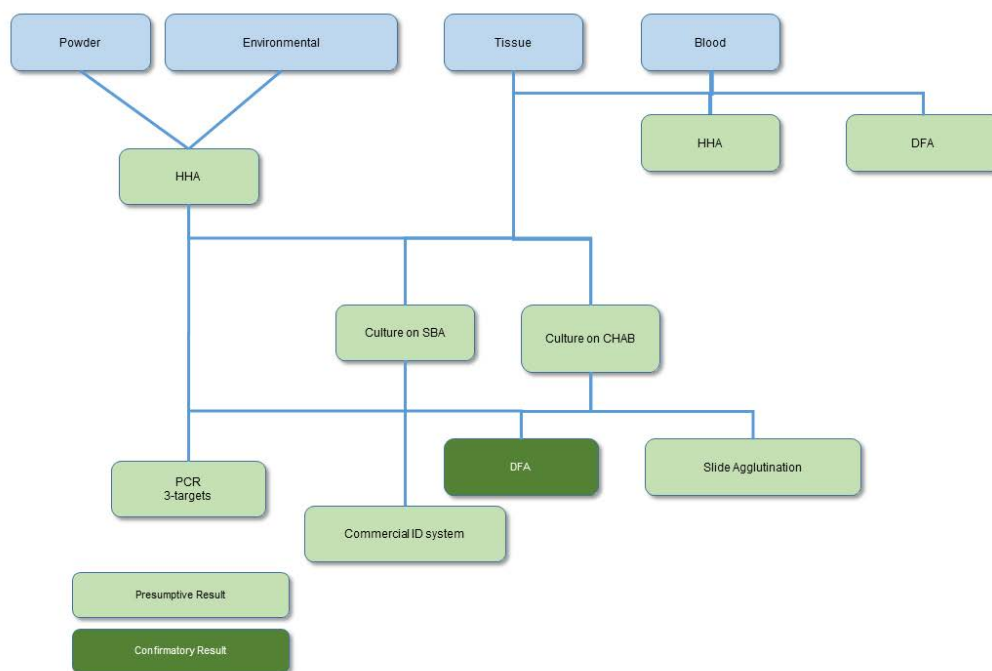
Multiple-locus variable-number tandem repeat analysis (MLVA) system: The use of 24 or 25 marker MLVA schemes have been developed and used for characterization studies. Like most systems, MLVA was able to differentiate the standard *Francisella* taxonomy including the two distinct subpopulations (A.I. and A.II) of *F. tularensis* subsp. *tularensis* (19). Multilocus variable-number tandem repeat analysis using three to six allelic markers (18, 40, 82) has also been used.

"Canonical SNPs" (canSNPs) use rare single nucleotide polymorphisms (SNPs) for characterizing *Francisella* spp. The initial 38 canSNP analysis of *Francisella* was refined later and uses a 17 molecular inversion probe microarray technology. This is the most current method for characterization of *Francisella* strains (48, 83, 90-92).

## Confirmation.

Presumptive identification of *F. tularensis* is achieved by the detection of a biological marker using a single test methodology (for example, hand-held assay (HHA)). A positive test result with any one of (i) DFA, (ii) IHC staining, (iii) slide agglutination test, (iv) PCR, or (v) single antibody titer by serologic test also provides a presumptive diagnosis. Fatty acid profiles may also be used to presumptively identify the organism as belonging to the *Francisella* genus (33).

Definitive identification or confirmation of *F. tularensis* requires a combination of techniques and assays. In civilian laboratories, culture of the organism is required as part of confirmation. Culture of the organism precludes false-positive results from non-specific interference and allows the evaluation of false-negative results in the context of the broad array of testing performed. A fourfold titer difference in acute- and convalescent-phase serum samples is considered definitive for serological confirmation. Definitive identification or confirmation testing is performed at sanctioned reference laboratories, including reference laboratories of the CDC Laboratory Response Network (LRN). Specific LRN protocols and reagents are proprietary but any definitive identification or confirmation typically follows a general scheme (85):



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# Brucella

## OVERVIEW

Brucellosis is an important disease of livestock in many countries and is caused by infection with one of several species of *Brucellae*, a group of gram-negative cocco-bacillary, facultative intracellular pathogens. *Brucella* spp. most commonly associated with human infection includes *B. abortus*, *B. melitensis*, *B. suis*, and rarely *B. canis*. In livestock, depending on the species affected, brucellosis is associated with infertility, abortion, retained fetal membranes, orchitis, and infection of the male accessory sex glands. Transmission in most livestock is primarily via ingestion but they can also readily enter the body through mucous membranes, conjunctivae, wounds, and occasionally through intact skin. Transmission to humans occurs typically by ingesting raw milk and other dairy products from infected animals but airborne infections have also occurred in livestock, husbandry settings, and in the laboratory. It is estimated that inhalation of only 10 to 100 bacteria is sufficient to cause disease in humans. The various strains of *Brucella* causes the disease called "brucellosis" "Bang's disease", "Malta fever", "undulant fever", and a host of other disease names.

Depending on the taxonomy being used, *Brucellae* contain 10 recognized species that include *B. abortus*, *canis*, *melitensis*, and *suis*, the most common and important human pathogens. Differentiating the human pathogenic species from the other *Brucellae*, however, is not easy and requires several growth and biochemical determinations. Immunoassay and nucleic acid assays are currently not helpful in distinguishing the pathogens from the non-pathogens. Culture is required for confirmation.

## ORGANISM INFORMATION

### Taxonomy Information.

The *Brucella* genus contains 10 recognized species, some with biovars. The first human cases of brucellosis were described in 1861 by J. A. Marston, a physician with the British army stationed at Malta (13, 42). The causative agent of brucellosis was first cultivated in 1886 by Sir David Bruce from spleen tissues of victims of Malta fever. In 1895, Bang, a Danish veterinarian, isolated an organism, which he designated *Bacillus abortus*, from cases of bovine abortion. The three species were combined in the *Brucella* genus in 1918. In 1929, a third species, *B. suis*, to include strains from aborted swine. Two additional species were identified in the 1950s: *B. ovis*, an agent of reproductive disease in sheep, and *B. neotomae*, isolated from the desert wood rat in Utah, neither of which has been associated with human disease (64). In 1966 a sixth *Brucella* species was added, *B. canis*, which is a causative agent of canine abortion (14). Over the past 10 years, multiple studies have described phenotypically unique *Brucella* strains isolated from marine mammals (*B. pinnipedialis* and *B. ceti*) (9, 20, 31, 67), as well as a novel *Brucella* from a breast implant (*B. inopinata*) (17).

The taxonomy of *Brucella* was muddled in 1986 when some taxonomists changed the *Brucella* taxonomy to have only one species (*Brucella melitensis*) with 18 biovars, but the separation back to the current 10 species was ratified in 2008 and upheld again in 2010. The original species and biovars were defined by their host specificity. However, DNA relatedness and multilocus enzyme electrophoresis studies indicate that all these organisms, along with the recent marine mammal-derived strains, represent a single species. Molecular taxonomic studies showed that a high overall level of relatedness exists and species can be differentiated by restriction polymorphisms of major outer membrane genes, insertion sequences, and whole-chromosomal preparations. **Some organizations are still using the 1986 nomenclature**, which is causing confusion.

- a. *Brucella abortus*
  - (1) *Brucella abortus* biovar 1
  - (2) *Brucella abortus* biovar 2
  - (3) *Brucella abortus* biovar 3
  - (4) *Brucella abortus* biovar 4
  - (5) *Brucella abortus* biovar 5
  - (6) *Brucella abortus* biovar 6
  - (7) *Brucella abortus* biovar 7 [Work is ongoing on to clarify the taxonomy of biovar 7.]
  - (8) *Brucella abortus* biovar 8; Deleted by Subcommittee on the Taxonomy of *Brucella* (1978)
  - (9) *Brucella abortus* biovar 9

- b. *Brucella canis*
- c. *Brucella ceti*
- d. *Brucella inopinata*
- e. *Brucella melitensis*
  - (1) *Brucella melitensis* biovar 1
  - (2) *Brucella melitensis* biovar 2
  - (3) *Brucella melitensis* biovar 3
- f. *Brucella microti*
- g. *Brucella neotomae*
- h. *Brucella ovis*
- i. *Brucella pinnipedialis*
- j. *Brucella suis*
  - (1) *Brucella suis* biovar 1
  - (2) *Brucella suis* biovar 2
  - (3) *Brucella suis* biovar 3
  - (4) *Brucella suis* biovar 4
  - (5) *Brucella suis* biovar 5

### Virulence Factors.

Brucellosis is principally a disease of animals with human infection as secondary infections. *Brucellae* are facultative intracellular organisms and the disease spectrum is partially explained by the ability of the organism to evade host defense mechanisms by virtue of its intracellular existence. Virulence factors for *Brucella* have not been especially well established, but several putative ones have been identified. Like other gram-negative, intracellular bacteria, a number of factors have been proposed as virulence factors, but there are not any especially unique *Brucella* virulence factors. In addition, some of the virulence factors identified were identified by the differences in virulence between a fully functional system and a mutated system. That premise makes an indirect association between a mutation and a loss of virulence, not a direct one (the mutation may be in an accessory system that feeds or regulates another factor that is specifically the virulence factor) (48).

*Brucella* lipopolysaccharide (LPS): The LPS of *B. melitensis*, *B. abortus*, and *B. suis* contains two major antigenic determinants called A (for "abortus") and M (for "*melitensis*"). The A and M antigenic determinants are not found in *B. canis*. These determinants are not species-restricted, and both may be expressed to varying degrees on the same cell. As a result, a great deal of serologic cross reactivity may be seen among these three species. In culture, *Brucella* undergoes antigenic variation or "dissociation." Morphologically, the colonies switch from a "smooth" to a "rough" morphology, resulting in a loss of virulence and diminished reactivity with *Brucella*-specific antibodies. The antigenic variation is the result of decreased expression of genes encoding the additional glycosylation of the polysaccharide moieties of the cell wall lipopolysaccharide (LPS). Organisms that are in the smooth phase are resistant to intracellular killing by polymorphonuclear cells (PMNs), presumably by inhibiting degranulation in the lysosome and the respiratory burst associated with activation of PMNs.

Very little else is known about additional virulence mechanisms in *Brucella* species though the following have also been cited as virulence factors without useful details:

- Capsules.
- Fimbriae/flagella.
- Exotoxins.
- Exoproteases or other exoenzymes.
- Cytolysins.

Disease type and severity vary with the infecting strain of *Brucella* without a direct correlation to a particular virulence factor at this time:

*B. melitensis* is the most human pathogenic strain; human infection is associated with an acute course with disabling complications.

*B. suis* infection is associated with localized abscess formation and a chronic course.

*B. abortus* and *B. canis* infections are associated with frequent relapses and insidious onset.

### Epidemiology and Endemic Areas.

Brucellosis in humans is typically an acquired disease from infected domestic, or wild, animals. Human-to-human transmission is rare. Consumption of contaminated animal tissues, to include milk products, contact with infected animal tissues, and laboratory infection are the most common causes of

Brucellosis in humans.

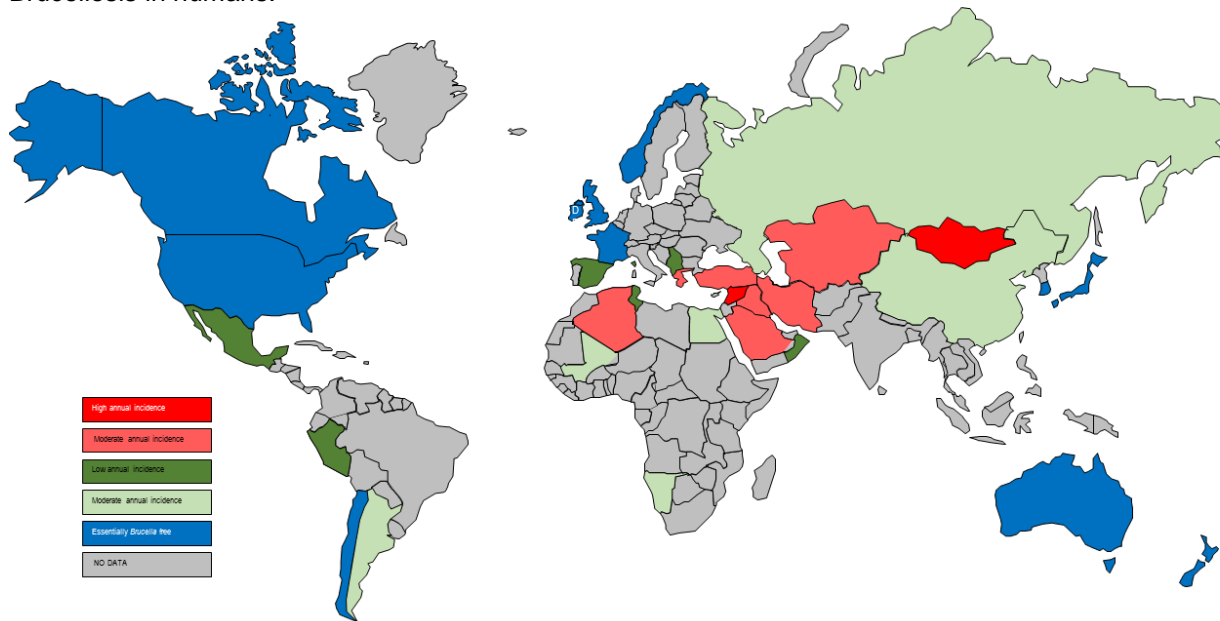


Figure 33. Generalized international incidence of Brucellosis.

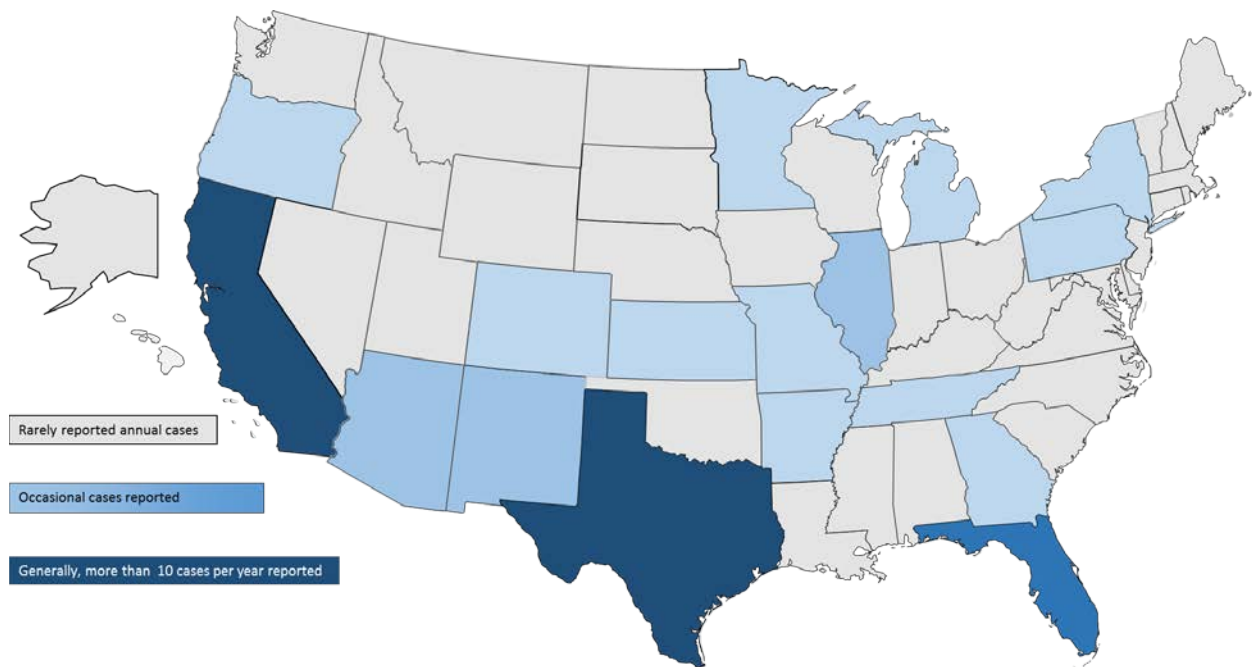


Figure 34. Historical brucellosis status by state. CDC does not keep specific *Brucella* spp. information on infections. Most states are *B. abortus* free for cattle and all states, except Texas are *B. suis* free (2009 data last reported) for swine. *B. abortus* remains enzootic in elk and bison in the greater Yellowstone National Park area, and *B. suis* is enzootic in feral swine in the Southeast. California, Texas, and Florida continuously have the most brucellosis reports on an annual basis.



## LABORATORY DIAGNOSTICS

### Biosafety Information.

Biosafety in Microbiological and Biomedical Laboratories (BMBL) Containment Recommendations (5th Edition): Biosafety level (BSL)-2 practices, containment equipment, and facilities are recommended for routine clinical specimens of human or animal origin. Products of conception containing or believed to contain pathogenic *Brucella* should be handled with BSL-3 practices due to the high concentration of organisms per gram of tissue. BSL-3 and animal BSL-3 practices, containment equipment, and facilities are recommended, for all manipulations of cultures of pathogenic *Brucella* spp. listed in the BMBL, and for experimental animal studies.

### Diagnostic Information.

#### General.

*Brucellae* are gram-negative, non-motile, non-encapsulated, non-spore forming, aerobic, slow-growing coccobacilli or short rods measuring from 0.6 to 1.5 µm long and from 0.5 to 0.7 µm wide. They are usually arranged singly, and less frequently in pairs or small groups. Older cultures may show pleomorphic forms.

#### Staining

*Brucella* are gram-negative though hard to visualize individual cells. Direct microscopic examination of blood or bone marrow is not sufficiently sensitive to be useful in the diagnosis of brucellosis.

Ziehl–Neelsen's acid-fast stain: They are not truly acid-fast, but are resistant to decolorization by weak acids and thus stain red by the Koster's (safranin staining followed by NaOH decolorization), Macchiavello's (basic fuchsin staining followed by decolorization nitric acid decolorization) or Stamp's (basic fuchsin staining followed by decolorization with diluted acetic acid) modification of the Ziehl–Neelsen's method. Ziehl–Neelsen's method is the usual procedure for the examination of organs or biological fluids smears that have been previously fixed with heat or ethanol. *Brucella* organisms stain red against a blue background. *B. ovis* are not positive (red) with the Koster's acid-fast modification.

Direct immunofluorescence assays (DFA): Direct immunofluorescence assays (DFA) have been used (see "Immunoassays" section).

#### Metabolic Information

All *Brucella* are aerobic but four biovars of *B. abortus* need increased CO<sub>2</sub> for growth. *Brucella* are metabolically slow organisms and typically require 3 to 7 days before colonies are visible on agar medium; sometimes longer. While *Brucella* can produce acids from carbohydrates, the metabolism is predominately oxidative (56). *Brucella* are catalase positive, most are oxidase positive, and most produce hydrogen sulfide from amino acids. Urease is produced by most strains with some strains producing a lot and others being weaker.

Optimal temperature: 35-37°C.

Upper temperature: 40°C

Lower temperature: 20°C.

Optimal pH: The optimum growth pH ranges from 6.6 to 7.4.

#### General Culturing Information

Growth is obtained on most commonly used media including trypticase soy (with and without 5% sheep blood), *Brucella*, brain heart infusion, heart infusion, MacConkey agar (variable), Martin Lewis agar, and chocolate agars. Enhanced CO<sub>2</sub> may be required for growth of some strains, especially on primary isolation. Growth is improved by serum or blood, but hemin (X factor) and NAD (V factor) do not contribute to enhance growth. When grown on blood agar at optimal temperature and atmosphere, colonies are usually 0.5 to 1.0 mm in diameter, raised, and convex, with an entire edge and a smooth shiny surface. *B. canis* and *B. ovis* characteristically produce non-smooth colonies. Non-smooth variants of the other species also occur (see discussion in virulence factors). Optimal incubation temperatures are 35-37°C for 3-7 days,



Figure 35. *Brucellae* on sheep blood agar, demonstrating the typical pin-point colony size.

High annual incidence

continued incubation for upto 21 days may be necessary.

*Brucellae* grow in the aerobic component of essentially all blood culture systems, trypticase soy agar with 5% sheep blood agar, MacConkey agar, or Martin Lewis agar, although differences exist in the time of detection and sensitivity depending upon the system used. Biphasic bottles (Castenada bottles) containing brain heart infusion broth with a 2.5% agar slants portion are considered the method of choice (71). Blood culture bottles are inoculated with blood to a final concentration of 10% (vol/vol) and incubated under 10% CO<sub>2</sub>, tension for up to 6 weeks at 35 to 37°C. At 3-day intervals the bottles are tilted to allow the inoculated broth to wash over the agar slant and then returned to the upright position. Growth, as indicated by turbidity in the broth or formation of colonies on the agar, is monitored visually on a daily basis. Various studies have reported times to detection ranging from less than 6 days to more than 27 days for biphasic bottles.

Note: Plate cultures should be incubated in 5% carbon dioxide as some *B. abortus* require CO<sub>2</sub> enrichment (most *Brucella* do not). It typically takes 3-7 days to form colonies on plates.

Lysis centrifugation (involving osmotic lysis of erythrocytes followed by concentration of bacteria by centrifugation and direct plating of the concentrate onto culture media) improves the sensitivity and time to detection of *Brucellae* compared with Castenada bottles, but the newer automated blood culture systems produced higher sensitivity rates (36).

#### *Brucella* Media.

*Brucella* agar was originally developed for the cultivation of *Brucella* spp. from diagnostic specimens, but the media have found wider, and more general, applications. *Brucella* agar with 5% horse blood plates are particularly useful for the cultivation of the more fastidious aerobic and anaerobic microorganisms, and is not limited to *Brucella* cultivation. Other variations include *Brucella* albimi broth with 0.16% agar and 1% glycine, *Brucella* anaerobic blood agar, *Brucella* broth, *Brucella* broth with 0.16% agar, *Brucella* medium base, *Brucella* Medium, *Brucella* selective *Brucella* agar, CTA medium, liver Infusion agar/broth, and potato infusion agar.

#### Selective culturing of *Brucella*.

Farrell's medium: Is prepared by the addition of six antibiotics to a basal medium. The antibiotics polymyxin B sulphate, bacitracin, natamycin, nalidixic acid, nystatin, and vancomycin are used. However, nalidixic acid and bacitracin, at the concentration used in Farrell's medium, have inhibitory effects on some *B. abortus* and *B. melitensis* strains. Therefore the sensitivity of culture increases significantly by the simultaneous use of both Farrell's and the modified Thayer–Martin medium.

#### Diagnostic Tests.

Generally, *Brucella* spp. are difficult to identify to the strain and biovar level. Strains are positive for urease, nitrate reductase, oxidase, and catalase, although weak reactions may be observed with some strains in the last two tests. Strains are negative for indole production, gelatin liquefaction, and hemolysis. The methyl red and Vogues Proskauer tests are negative (1).

Differentiation of <i>Brucella</i> from clinical near neighbors								
Test	<i>F. tularensis</i>	<i>Brucella</i> spp.	<i>Bartonella</i>	<i>Acinetobacte</i> spp.	<i>Psychrobacter phenylpyruvici</i>	<i>Oligella</i> spp.	<i>Bordetella bronchiseptica</i>	<i>Hemophilus influenzae</i>
Oxidase	—	+	—	—	+	+	+	V
Urea hydrolysis	—	+	—	V	+	+	+	V
Gram stain morphology	Tiny ccb	Tiny ccb	Thin rod	Broad ccb	Broad ccb	Tiny ccb	Thin rod	Small ccb
X or V factor requirement	—	—	—	—	—	—	—	+
Cysteine enhancement	+	—	—	—	—	—	—	—
Motility	—	—	—	—	—	V	+	—
ccb - coccobacillary								

Tolerance to the basic dyes fuchsin and thionin: Tolerance to the dyes thionin and basic fuchsin is determined by examining the growth on agar plates containing 1:25,000, 1:50,000, and 1:100,000 dilutions of dye. The dilutions are made by adding 0.8, 0.4, or 0.2 ml of 1% stock solutions to 20 ml of heart infusion agar that has cooled to 50°C before pouring. The inoculum is prepared by suspending a loopful of bacterial growth from a freshly grown culture into 1 ml of physiological saline. A sterile cotton

swab is immersed into the bacterial suspension and used to inoculate the dye plates and one control plate containing no dye. A single streak is made across each of the dye plates and, lastly, a control plate containing no dye. The plates are inverted and incubated at 35°C for up to 4 days (if increased CO<sub>2</sub> is required, the plates may be incubated in a CO<sub>2</sub> incubator or a candle jar) and examined for growth on a daily basis.

CO<sub>2</sub> requirement: A growth requirement for elevated CO<sub>2</sub> levels is observed in some strains of *B. abortus*, *B. ovis*, and *B. pinnipedialis*. This requirement is sometimes lost by multiple passages in the laboratory. The CO<sub>2</sub> requirement test is performed by streaking two plates of general-use media (trypticase soy agar with or without 5% sheep blood or heart infusion agar with or without 5% rabbit blood) and then incubating one plate in ambient air and the other plate in 5% CO<sub>2</sub>. Plates are examined at daily intervals, and the growth on the plates is compared.

Gel formation and acriflavin tests: The acriflavin and gel formation tests are useful in differentiating *B. canis* from other *Brucella* species. The acriflavin reagent is made at a concentration of 1 mg/ml in distilled water. The test is performed by placing a drop of reagent on a slide and mixing it with fresh growth of the test organism. Agglutination of the organisms is indicative of a positive test. The gel formation test is performed by making a heavy suspension of a fresh culture in a small volume of phenolized saline (0.5% phenol in buffered saline) in a 13- by 100-mm screw-cap test tube. The tube is allowed to stand at room temperature for 30 min and then is observed for gel formation. Gel formation can be determined by inserting a bacteriological loop in the suspension and looking for the formation of a short, mucoid string as the loop is withdrawn. Another technique for observing gel formation is tilting the tube and comparing the viscosity of the test suspension with that of buffered saline in a control tube. If no reaction is observed at 30 min, the tube may be incubated at room temperature overnight and observed the next day. *B. canis* strains are characteristically positive for these tests, whereas the other *Brucella* species are usually negative.

Rate of urease activity: A positive urease test is characteristic of the genus *Brucella*. *B. suis* and some *B. melitensis* strains produce a rapid reaction that can be observed within 5 min of inoculation on a Christensen's urea slant. The other *Brucella* spp. usually produce a positive reaction after overnight incubation. Other urease-positive species that may be confused with *Brucella* include *Psychrobacter phenylpyruvicus*, *Oligella ureolytica*, *Bordetella bronchiseptica*, and some *Hemophilus influenzae* biogroups (60).

Hydrogen sulfide (H<sub>2</sub>S) test: H<sub>2</sub>S testing is performed by inoculating a slant of *Brucella* or heart infusion agar with fresh growth of the test organism. After inoculation, a lead acetate paper strip is suspended above the slant so that the paper and the medium do not come in contact. The tube is incubated at 35°C and examined daily for 6 days. A positive reaction is indicated by the development of a dark gray or black color on the strip. Triple sugar iron agar (TSIA) slants will not work.

<b>Differentiation of <i>Brucella</i> species and biovars</b>								
<b>Species</b>	Biovar	CO <sub>2</sub>	H <sub>2</sub> S	Urease	Basic Fuschin	Thionin 20 ug	Thionin 40 ug	Thionin Blue
<b><i>B. melitensis</i></b>	1	—	—	V	+	+	+	+
	2	—	—	V	+	+	+	+
	3	—	—	V	+	+	+	+
<b><i>B. abortus</i></b>	1	w/v	+	1-2 h	+	—	—	+
	2	w/v	+	1-2 h	—	—	—	—
	3	w/v	+	1-2 h	+	+	+	+
	4	w/v	+	1-2 h	+/-v	—	—	+
	5	—	—	1-2 h	+	—	+	+
	6	—	+/-v	1-2 h	+	—	+	+
	7	—	+	1-2 h	+	—	+	+
<b><i>B. suis</i></b>	1	—	+	0-30 min	—/-v	+	+	—/-v
	2	—	—	0-30 min	—	—	+	—
	3	—	—	0-30 min	+	+	+	+
	4	—	—	0-30 min	—/-v	+	+	—
	5	—	—	0-30 min	—	+	+	NA
<b><i>B. canis</i></b>		—	—	0-30 min	—	+	+	—/-v
<b><i>B. ovis</i></b>		+	—	—	-/-v	+	+	—
<b><i>B. neotoma</i></b>		—	+	0-30 min	—	—	—	+

<i>B. ceti</i>	—	—	+	+	Not tested	Not tested
<i>B. pinnipedialis</i>	+	—	+	+	Not tested	Not tested
<i>B. microti</i>	—	—	+	+	Not tested	Not tested

Agglutination in monospecific adsorbed rabbit antiserum, anti-A, -M or -R, are used in a standard agglutination reaction. Different strains of *Brucella* spp. will react in biotype specific reactions.

Susceptibility to *Brucella* Tbilisi phage: Susceptibility/lysis of *Brucella* by the *Brucella* Tbilisi phage differentiates *B. melitensis* from *B. abortus* and *B. suis*. Tbilisi phage is commercially available from the American Type Culture Collection (ATCC 23448-BI). The routine test dose (RTD) is determined by testing 10-fold dilutions of stock phage suspension against a control *B. abortus* strain, such as ATCC 23448. The highest dilution at which a complete plaque is observed is the RTD. The test is performed with a low concentration of cells, approximately 10 cells per ml, and then inoculating agar plates to produce a uniform lawn on the plate. After a few minutes to allow absorption of the inoculum, 10- $\mu$ l volumes of the RTD and RTD x 10<sup>4</sup> are spotted on the plate. Plates incubated at 35°C and read on a daily basis for 2 days. CO<sub>2</sub>-requiring isolates should be incubated in a 5% CO<sub>2</sub>, incubator or a candle jar. The simultaneous use of several phages, e.g., Tbilissi (Tb), Weybridge (Wb), Izatnagar (Iz) and R/C (22), provides a phage-typing system that, in experienced hands, allows a practical identification of smooth and rough species of *Brucella*.

Differential characteristics of species of the genus <i>Brucella</i> by phages						
	Colony morphology (S: smooth, R: rough)	Tbilisi (Tb)		Weybridge (Wb)	Izatnagar1(Iz1)	R/C
		RTD	10 <sup>4</sup> RTD	RTD: routine test dilution		
<i>B. abortus</i>	S	+	+	+	+	—
<i>B. suis</i>	S	—	+	+a	+a	—
<i>B. melitensis</i>	S	—	—	—b	+	—
<i>B. neotomae</i>	S	—c	+	+	+	—
<i>B. ovis</i>	R	—	—	—	—	+
<i>B. canis</i>	R	—	—	—	—	+
<i>B. ceti</i>	S	+d		+e	+f	—
<i>B. pinnipedialis</i>	S	+d		+e	+f	—
<i>B. microti</i>	S	—	+	+	+	—

a Some isolates of *B. suis* biovar 2 are not or partially lysed by phage Wb or Iz1  
b Some isolates are lysed by phage Wb  
c Minute plaques  
d Some isolates are lysed by Tb  
e Most isolates are lysed by Wb  
f Most isolates are lysed by Iz

#### Identification of vaccine strains

Identification of the vaccine strains of *B. abortus* is important, both in field investigations, as well as for select agent determinations. *B. abortus* S19, *B. abortus* RB51 and *B. melitensis* strain Rev.1, are exempt from select agent requirements. *B. abortus* S19 has the normal properties of a biovar 1 strain but does not grow in the presence of benzylpenicillin (3  $\mu$ g/ml = 5 IU/ml) and has a high L-glutamate use. In some cases S19 will grow in the presence of erythritol, but does not use it (35). *Brucella melitensis* strain Rev.1 has the normal properties of a biovar 1 strain of *B. melitensis*, but develops smaller colonies on agar media, does not grow in the presence of basic fuchsin, thionin (20  $\mu$ g/ml) or benzylpenicillin (3  $\mu$ g/ml), but does grow in the presence of streptomycin at 2.5 or 5  $\mu$ g/ml. *B. abortus* strain RB51 is identified by a rough colony morphology and growth in the presence of rifampicin (250  $\mu$ g/ml). Vaccine strains S19, Rev.1 and RB51 may also be identified using specific PCRs (12, 8).

#### Commercial Identification systems

(see Appendix E for technical descriptions of the methods)

NOTE: Rapid identification systems may mis-identify the organism, often as *Psychrobacter phenylpyruvicus*, *Ochrobactrium* spp. or *Roseomonas* spp.

Fatty acid methyl ester (FAME) analysis: Cellular fatty acid (CFA) profiles are also very useful in differentiating *Brucella* from other similar taxa. The *Brucella* CFA profile is characterized by significant amounts of capric, myristic, palmitic, oleic, and phydroxystearic acids. *Brucella* spp., except *Brucella canis* (18), are characterized by large amounts of fatty acid 19:0.

- (1) The commercial library databases (BTR3, RBTR3, CLIN6, and RCLIN6) lack enough *Brucella* data to identify all the possible identifications, either due to time of incubation or media.
- (2) Culturing for 24 hr was most efficient for identification, based on current database entries for *Brucella*, but a 24 hr culture but cannot always yield sufficient cell mass for analysis, especially using the standard method.
- (3) Use of 48 hr culture growth yields sufficient cell mass, but are not as accurate for identification. The commercial database does not have enough profile information to provide identifications for the 48 hr fatty acid profiles that are produced.
- (4) The fatty acid profile for *Brucella* that could be found in all the profiles tested, contain fatty acids 16:0, 18:0, "sum in Feature 8", "summed Feature 8", and 20:1 (w7c). The current commercial database does not incorporate 20:1 (w7c). This profile was seen in all growth conditions to detect *Brucella* spp. on the genus level and could differentiate *Brucella* from *Ochrobactrium* and *Roseomonas*. Using this profile of peak names, misidentifications with *Methylobacterium*-, *Ochrobactrium*-, and *Roseomonas*-specific fatty acids can be avoided since the profile was unique to *Brucella* species.

Vitek: The original Vitek (32 chemical reactions) did not have *Brucella* spp. in the database but the Vitek2 does. Using the Vitek2 with the GN2 card, 100% of test strains were correctly identified as *Brucella melitensis* or *Brucella* (16). The *Brucella melitensis* identification of the other species of *Brucella* reflects the difference of terminologies in existence. From internal unpublished information, while the identification of the *Brucella* spp. is not possible, at least the correct genus seems to be consistently identified. Regardless of results, confirmation of Vitek identifications should be done.

Biolog: While the GN2 system had a Dangerous Pathogens database for identification of *Brucella*, the current GENIII does not. The Biolog OmniLog® Dangerous Pathogen Database (version 6.12) is based on a monospecific genus in which all *Brucella* are identified as a single species of *B. melitensis*. One study (70) found that *B. melitensis*, *B. abortus*, and *B. suis* could be distinguished from each other on the basis of seven oxidation reactions, but internal studies were not able to identify many of the *Brucella* spp. tested (only 6 of 35). Biolog data showed that all *Brucella* had strong positives for L-arabinose, pyruvic acid methyl ester, and succinic acid mono-methyl ester and various other reactions based on grouping of *Brucella* that does not match directly their species (GN2 data). *Brucella* spp. were able to be grouped into 7 different groups based on their carbon utilization patterns though the groups did not match current taxonomics.

Carbon Source	Group 1 <sup>i</sup>	Group 2 <sup>k</sup>	Group 3 <sup>c</sup>	Group 4 <sup>c</sup>	Group 5 <sup>c</sup>	Group 6 <sup>l</sup>	Group 7 <sup>g</sup>
Acetic acid	—	—	—	—	+	—	—
α-D-Glucose	—	—	—	+	—	+	+
Adonitol	—	—	—	—	+	—	—
α-Hydroxybutyric acid	—	+	—	—	+	—	—
α-ketobutyric acid	—	+	—	—	+	—	—
α-ketoglutaric acid	—	+	—	—	—	—	—
β- Hydroxybutyric acid	—	+	—	—	—	—	—
D,L-Lactic acid	—	+	—	—	+	—	+
D-Alanine	—	—	+	—	+	—	—
D-Fructose	—	—	+	+	—	—	—
D-Galactose	—	—	—	—	+	—	—
D-Gluconic acid	—	—	—	—	—	—	+
D-Mannitol	—	—	—	—	—	+	+
D-Psicose	—	—	—	—	+	—	—
D-Serine	—	—	+	—	—	—	—
Formic acid	—	—	—	—	+	—	—
Gentiobiose	—	—	—	—	—	+	+
Glucuronamide	—	—	—	—	—	—	+
Glycl-L-Glutamic Acid	—	+	—	—	—	—	—
i-Erythritol	—	+	+	+	—	—	—
Inosine	—	—	—	—	+	—	—
L-Alaninamide	—	—	+	—	+	—	—
L-Alanine	—	+	+	—	+	+	+



Carbon Source	Group 1 <sup>a</sup>	Group 2 <sup>k</sup>	Group 3 <sup>c</sup>	Group 4 <sup>c</sup>	Group 5 <sup>e</sup>	Group 6 <sup>l</sup>	Group 7 <sup>g</sup>
L-Alanyl-Glycine	—	—	—	—	—	+	+
L-Arabinose	+	—	—	—	+	—	—
L-Asparagine	—	—	—	—	+	—	—
L-Aspartic acid	—	—	—	—	—	+	+
L-Fructose	—	—	+	—	—	—	—
L-Glutamic acid	—	+	+	—	+	—	—
L-Leucine	—	—	—	—	—	+	—
L-Proline	—	—	+	—	—	+	+
m-Inositol	—	—	—	—	—	—	+
Pyruvic Acid Methyl Ester	+	+	+	+	—	—	—
S-Serine	—	—	—	—	—	—	—
Succinamic acid	—	+	—	—	—	—	—
Succinic acid	—	+	—	—	+	—	—
Succinic Acid Mono-Methyl Ester	+	+	+	+	—	—	—
Uridine	—	—	—	—	+	—	—
Urocanic acid	—	—	—	—	+	—	+
<sup>a</sup> - <i>B. suis</i> , n=8; <i>B. ovis</i> , n=1; <i>B. neotomea</i> , n=2; <i>B. abortus</i> , n=1							
<sup>b</sup> - marine isolates, n=3							
<sup>c</sup> - <i>B. melitensis</i> , n=18							
<sup>d</sup> - marine isolates, n=2; <i>B. melitensis</i> , n=1; <i>B. ovis</i> , n=1							
<sup>e</sup> - <i>B. abortus</i> , n=9							
<sup>l</sup> - <i>B. melitensis</i> , n=5							

### Immunoassay Tests:

NOTE: For a short explanation of the principles of the assay technologies, see Appendix C. The similarity of *Brucella* organisms presents a big problem for immunoassay based systems due to crossreacting antigenic proteins between the *Brucella* spp. and even other organisms. While the different Brucellosis diseases are present, testing for different *Brucella* spp., the immunoassay information is separated as best possible. Immunoassays for *Brucella* spp. also include the assays directed at animals.

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

Immunoassays have been developed to several immunoreactive components of the *Brucella* organisms. The *Brucella* OMP proteins produce a host response that is fairly specific for infection with *Brucella* organisms, but some cross reactions do occur. The OMP antigens with molecular masses of 10, 16.5, 19, 25 to 27, 36 to 38, and 89 kD and other proteins with molecular masses of between 40 and 80 kD have all been targeted for immunoassay, either individually or as a group. A competitive ELISA with the anti-89 kD monoclonal antibody was not specific. Results of the competitive ELISA confirmed the individual variability of the humoral immune response against OMPs. It therefore seems that a combination of several protein antigens is necessary for the development of an immunoassay with sensitivity comparable to that of the smooth lipopolysaccharide. The *Brucella* smooth and rough lipopolysaccharides also offer specific immunoassay targets for *Brucella* diagnostics.

#### Direct agent immunoassays.

Lateral flow immunochromatography (LFI): Hand-held assays (HHA), "Smart tickets", or lateral flow assays are commercially available and provide potentially useful (46).

Electrochemiluminescence (ECL): An ECL assay, based on the legacy M1M system, was developed for *Brucella* based on the OMP proteins as targets.

Serological testing. Serological testing for brucellosis has predominately been used on animals, but some human tests have been developed. Both are discussed below. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

Serum agglutination test (SAT): A SAT for IgM and IgG, and a tube agglutination method for anti-O polysaccharide antibody is available; titers of at least 1:160 by each indicate active disease (72). SAT

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is used commonly in the detection of both human and bovine *Brucella*- specific antibodies. The SAT is reported to have specificity between 95% and 100% and a sensitivity between 70% and 91.5%. The test can be used in a tube or microplate format and can be modified to differentiate IgG from IgM titers by pretreatment of specimens with 2-mercaptoethanol. The standard SAT method uses killed whole-cell *B. abortus* antigen and detects antibodies against *B. abortus*, *B. suis*, and *B. melitensis* but not *B. canis*. The typical fourfold rise in titer between the acute and convalescent-phase sample or a single titer of 2160 is suggestive of brucellosis. Cross reactions may be observed with antibodies directed against *Francisella tularensis*, *Vibrio cholerae*, or *Y. enterocolitica*. The SAT is regarded as being unsatisfactory for the purposes of animal international trade (5, 54).

**Brucellacapt:** Brucellacapt is a commercially available immunocapture agglutination test for the serodiagnosis of human brucellosis. Specificity (false positive) for the Brucellacapt test is reported to be between 81.5 % and 99.0%. Sensitivity (false negative) for the Brucellacapt test is reported to be 95.1% (54).

**Enzyme-linked immunosorbent assay (ELISA):** ELISA detects serum antibody and is reportedly able to distinguish between vaccine- and infection-derived antibodies. ELISA is used for detection of brucellosis in humans, cattle, sheep and goats. Specificity is reported to be between 60% and 96.5% and sensitivity to be between 94 % and 94.8% (19, 41).

**Coombs Test:** The Coombs Test is a diagnostic test using manufactured antigen and antiglobulins to detect the presence of antibodies. Antibodies bind to red blood cells that lyse in the presence of complement. It is used very commonly in the hospital blood banks and has found application in the detection of human brucellosis, but used less often to detect animal brucellosis. Specificity is reportedly between 96.2 % to 99.8% and sensitivity is reported to be about 91.5% (5, 54).

**Complement fixation test (CFT or CF):** The CFT is used to diagnose brucellosis in cattle and detects specific IgM and IgG1 antibodies. Specificity is reported to be about 98% and sensitivity is about 81%. CFT is diagnostically more specific than the SAT and also has a standardized system of values for reference (5).

For animal testing, various other serologic assays have been described, including the Rose Bengal test, Coombs anti-human globulin test, complement fixation test, and the rapid dipstick test. No single serological test is appropriate in all epidemiological situations; all have limitations especially when it comes to screening individual animals.

**Radial immunodiffusion (RID):** A radial immunodiffusion (RID) test uses manufactured *Brucella* antigens in a gelling agent with wells for serum. Sera positive for antibodies to *Brucella* will diffuse into the gelling agent and cause a visible precipitation or color change. RID tests are reported to have about 95% specificity and 100% specificity (19).

**Counter immunoelectrophoresis (CIEP):** Antigen binds with antibodies present in sera and the combination is electrophoresed to analyze antibody titers. CIEP is reported to have a specificity of about 90% and sensitivity of 93% (19).

**Milk Ring test (MRT):** MRT is a serological test for anti-*Brucella* IgM and IgA bound to milk fat globules in cow or goat milk. False positives may occur with this test in colostrum, milk at the end of a lactation period, or cows suffering from a hormonal disorder or mastitis; however the specificity is reported to be about 99% a sensitivity of about 56% (5).

**Rose Bengal test (RB):** The RB test is a spot agglutination technique that uses dyed *B. abortus* antigen to detect serum antibodies of bovine brucellosis. Specificity is reported to be between 71-80% with a sensitivity between 78-100% (5, 19).

**Skin Delayed-Type Hypersensitivity test (SDTH):** The SDTH test uses manufactured brucellin to elicit skin hypersensitivity in livestock infected with acute, chronic, or latent brucellosis. Specificity of the SDTH test is reported to be 93.9% (5).

**Lateral flow immunoassay:** A dipstick assay for rapid detection of *Brucella* specific immunoglobulin uses *Brucella* antigen on a nitrocellulose strip. When incubated for 3 h with a serum sample, positive samples will form a distinct line, which can be graded from 1-4. Specificity of the dipstick assay is reported to be 98.6% and sensitivity is reported to range from 89.0% at 0-2 months after the onset of the disease to 29.8% at 6 or more months after the onset of the disease. The rapid dipstick assay can be used as a field test (63).

**Fluorescence polarization assay (FPA)** (a prescribed test for international animal trade): As the test antigen, a small molecular weight fragment (average 22 kD) of *B. abortus* strain 1119-3 LPS is used. The antigen is labeled with fluorescein isothiocyanate (FITC). The antigen is added to diluted serum or whole blood and fluorescence change is monitored on a fluorescence polarization instrument. Serum is diluted and an initial reading to assess light scatter is obtained followed by the addition of the labeled antigen. Antibodies will bind the antigen changing the conformation of the fluorescent signal. A reading (in



milli- polarization units, mP) over the established threshold level is indicative of a positive reaction. The test should be calibrated against International Standard reference sera when used for international trade. Control sera of strong positive, weak positive and negative, as well as S19 vaccinate serum, should be included (51, 52).

### Nucleic Acid Detection Tests.

*Brucellae* contains two circular chromosomes and is not known to contain any naturally occurring plasmids (34, 45).

PCR targets have been developed by a number of researchers all trying to develop specific and sensitive assays:

*Brucella*-specific outer membrane proteins based PCR assay include:  
(4, 10, 15, 24, 38, 43, 44, 50, 49, 53, 58, 57, 59, 61, 62, 73)

- omp2a, omp2b, 36-38 kD OMPs or group 2 porin proteins [966-1128 bp].
- omp25, 25-27 kD OMPs, group 3 cell envelope proteins [642 bp].
- omp31, 31-34 kD OMPs, group 3 proteins [786 bp].
- Perosamine synthetase (*per*) gene (6).
- Insertion sequence gene IS711 found in all species of *Brucella* (resulting in a 52-bp reacting with 14 strains of *Brucella* from five species; (3)) and IS6501 (55).

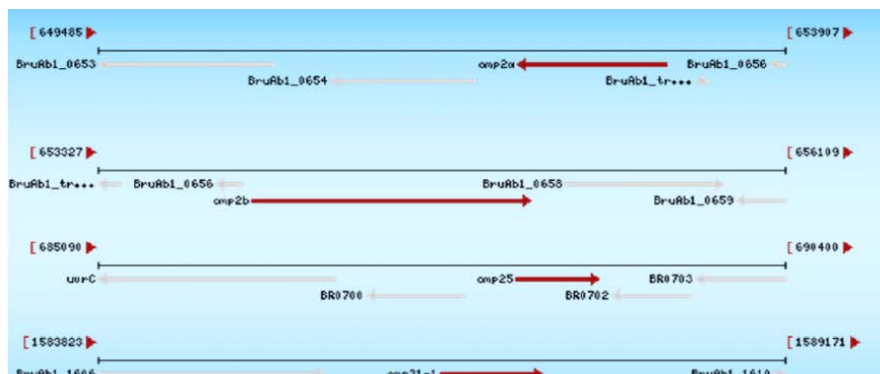


Figure 36. Cartoon of the omp genes being targeted by nucleic acid detection assays.

AMOS: Probably the best-established molecular method for identification of *Brucella* is the AMOS PCR assay, originally developed by the U.S. Department of Agriculture in 1994 (7). This assay, which takes advantage of species-specific locations of the insertion element IS711 (primer size 178bp), has been used to differentiate among three biovars of *B. abortus*, all biovars of *B. melitensis* and *B. ovis*, and biovar 1 of *B. suis*. The AMOS assay has been modified to differentiate the *B. abortus* vaccine strains RB-51 and S19 from wild-type strains and has been adapted to Lightcycler real-time detection technology (8). Although this assay identifies the biovars most commonly encountered in the U.S. agricultural sector, it does not identify *B. canis* and many of the *B. suis* biovars (12, 21).

Bruce-ladder: Bruce-ladder is a multiplex PCR assay that can detect and differentiate among several of the *Brucella* species, including the vaccine strains *B. abortus* RB51, *B. abortus* B19 and *B. melitensis* Rev.1. Bruce-ladder uses eight pairs of primers and after PCR, the amplicons are separated by gel electrophoresis. Different profiles differentiate among *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae*, the vaccine strains RB51, B19 and Rev.1 and the marine mammals isolates. Bruce-ladder was evaluated with more than 600 *Brucella* isolates from different geographic origins and different animal species included humans. It is commercially available. The PCR Bruce-ladder (27, 40, 39) has been recommended by the OIE (World Organization for Animal Health) in the chapter about bovine brucellosis 2009.

Joint Biological Agent Identification and Diagnostic System (JBAIDS). This *Brucella melitensis* (Brucellosis) detection kit, when used with the JBAIDS platform (hardware and software), is capable of detecting *Brucella melitensis* organism. Specifically, this Brucellosis test is based on a PCR assay that recognizes DNA target sequences present in *Brucella melitensis*. For optimal assay performance, the DNA of the pathogen has to be initially isolated from specimens using appropriate sample purification kits and procedures. This PCR-based test, however, does not distinguish between DNA, live or dead organisms.

### Other Diagnostic Methods.

None identified.

### Characterization.

With the high degree of DNA homology within the genus *Brucella*, species and strain characterization are difficult. Several molecular methods, including PCR (26), PCR restriction fragment length polymorphism (RFLP), and Southern blot, have been developed that allow, to a certain extent, differentiation between *Brucella* species and some of their biovars (47). Random amplified polymorphic DNA with 10-base primers (66) and restriction analysis of the omp2 locus after PCR amplification have been tried to differentiate strains of *Brucella* species (62).

Additional characterization techniques used include:

- Pulse-field gel electrophoresis has been developed that allows the differentiation of several *Brucella* species (32, 33).
  - Multiple-locus variable number tandem repeat analysis (MVLA/VNTR) has been tried (2, 11, 28, 29, 37, 69).
- Amplified fragment length polymorphisms (AFLP) (23, 68).
- Automated ribotyping (30).
- repPCR (65).
- Single nucleotide polymorphisms (SNPs) (25)

Definitive characterization methods for *Brucellae* are still needed as all the current methods have flaws that prevent wide spread adoption, leaving individual researchers to continue to use their 'favorite' method.

### Confirmation.

Presumptive identification of *Brucella* spp. is achieved by the detection of a biological marker using a single test methodology (for example, hand-held assay [HHA]). Presumptive evidence of *Brucella* is provided by the demonstration, by modified acid-fast staining of organisms, of *Brucella* morphology in abortion material or vaginal discharge, especially if supported by serological tests. The polymerase chain reaction methods provide additional means of detection. Whenever possible, *Brucella* spp. should be isolated using plain or selective media by culture from uterine discharges, aborted fetuses, udder secretions or selected tissues, such as lymph nodes and male and female reproductive organs. Species and biovars should be identified by phage lysis, and by cultural, biochemical, and serological criteria. PCR can provide both a complementary and biotyping method based on specific genomic sequences.

Definitive identification or confirmation of *Brucella* spp. requires a combination of techniques and assays, including most specifically, the culture of the organism to which testing can be performed directly on the organism. Culture of the organism precludes false-positive results from non-specific interference and allows the evaluation of false-negative results in the context of the broad array of testing performed. Definitive identification or confirmation testing is performed at sanctioned reference laboratories, including reference laboratories of the CDC Laboratory Response Network (LRN). Specific LRN protocols and reagents are proprietary but any definitive identification or confirmation typically follows a general scheme. Diagnostic laboratory criteria include: 1) isolation of *Brucella* sp. from a clinical specimen; 2) at least a fourfold rise in *Brucella* sp. agglutination titer between acute and convalescent sera obtained at least 2 weeks apart and studied at the same laboratory; 3) demonstration by immunofluorescence of *Brucella* sp. in a clinical specimen.



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# Burkholderia

## OVERVIEW

*Burkholderia mallei* and *Burkholderia pseudomallei* are the causative agents of glanders and melioidosis respectively. Both are oxidase positive, gram-negative saprophytic bacilli. *B. mallei* is primarily noted for producing disease in horses, mules, and donkeys where-as *B. pseudomallei* is a deadly human disease. In the past, humans have seldom been infected with *B. mallei*, despite frequent and often close contact with infected animals. The lymphatic thickening and induration seen in infected horses is known as “farcy” and human cases have occurred primarily in veterinarians, horse and donkey caretakers, and abattoir workers. Melioidosis, caused by *B. pseudomallei*, is present in water and soil in tropical and subtropical regions (1) and is spread to humans through direct contact or inhalation with the contaminated material. Because of *B. pseudomallei*'s potentially long incubation period, French and U.S. soldiers returning from Viet Nam would sometimes develop disease years later. *B. pseudomallei*, like *B. mallei*, was studied by the U.S. as a potential biowarfare agent, but was never weaponized. It has been reported that the former Soviet Union was experimenting with *B. pseudomallei* as a biowarfare agent.

*B. mallei* and *B. pseudomallei* are two of the 60 currently recognized species that include other human pathogens. As part of their environmental saprophytic lifestyle, the *Burkholderia* are complex organisms that are readily culturable but often display colony morphology variations that confound routine microbiological analysis. Biochemical differentiation, including gentamicin and polymyxin susceptibility, determination of arginine dihydrolase and lysine decarboxylase, and arabinose fermentation are required for differentiation and confirmation.

## ORGANISM INFORMATION

### Taxonomy Information.

*Burkholderia* consists of about 86 currently designated strains (2015), of which ***Burkholderia mallei***, ***Burkholderia pseudomallei***, and ***Burkholderia cepacia***, are the important human pathogens.

1. **Whitmore A, Krishnaswami CS.** 1912. An account of the discovery of a hitherto undescribed infective disease occurring among the population of Rangoon. Indian Med Gaz:262-267.

<i>Burkholderia</i>	<i>Burkholderia</i>	<i>Burkholderia</i>
<i>acidipaludis</i>	<i>contaminans</i>	<i>jiangsuensis</i>
<i>Burkholderia</i>	<i>Burkholderia</i>	<i>Burkholderia</i>
<i>ambifaria</i>	<i>cordobensis</i>	<i>kururiensis</i>
<i>Burkholderia</i>	<i>Burkholderia</i>	<i>Burkholderia lata</i>
<i>andropogonis</i>	<i>denitrificans</i>	<i>Burkholderia latens</i>
<i>Burkholderia anthina</i>	<i>Burkholderia</i>	<i>Burkholderia mallei</i>
<i>Burkholderia arboris</i>	<i>diazotrophica</i>	<i>Burkholderia</i>
<i>Burkholderia aspalathi</i>	<i>Burkholderia diffusa</i>	<i>megapolitana</i>
<i>Burkholderia</i>	<i>Burkholderia dilworthii</i>	<i>Burkholderia metallica</i>
<i>bannensis</i>	<i>Burkholderia dolosa</i>	<i>Burkholderia</i>
<i>Burkholderia</i>	<i>Burkholderia eburnea</i>	<i>mimosarum</i>
<i>bryophila</i>	<i>Burkholderia</i>	<i>Burkholderia</i>
<i>Burkholderia</i>	<i>endofungorum</i>	<i>multivorans</i>
<i>caballeronis</i>	<i>Burkholderia ferrariae</i>	<i>Burkholderia nodosa</i>
<i>Burkholderia</i>	<i>Burkholderia</i>	<i>Burkholderia</i>
<i>caledonica</i>	<i>fungorum</i>	<i>norimbergensis</i>
<i>Burkholderia</i>	<i>Burkholderia</i>	<i>Burkholderia</i>
<i>caribensis</i>	<i>ginsengisoli</i>	<i>oklahomensis</i>
<i>Burkholderia</i>	<i>Burkholderia gladioli</i>	<i>Burkholderia oxyphila</i>
<i>caryophylli</i>	<i>Burkholderia glathei</i>	<i>Burkholderia</i>
<i>Burkholderia</i>	<i>Burkholderia glumae</i>	<i>phenazinium</i>
<i>cenocepacia</i>	<i>Burkholderia graminis</i>	<i>Burkholderia</i>
<i>Burkholderia cepacia</i>	<i>Burkholderia</i>	<i>phenoliruptrix</i>
<i>Burkholderia choica</i>	<i>grimmiae</i>	<i>Burkholderia</i>
<i>Burkholderia</i>	<i>Burkholderia heleia</i>	<i>phymatum</i>
<i>cocovenenans</i>	<i>Burkholderia hospita</i>	<i>Burkholderia</i>
	<i>Burkholderia humi</i>	<i>phytofirmans</i>

<i>Burkholderia pickettii</i>	<i>Burkholderia</i>	<i>Burkholderia terricola</i>
<i>Burkholderia plantarii</i>	<i>seminalis</i>	<i>Burkholderia</i>
<i>Burkholderia</i>	<i>Burkholderia</i>	<i>thailandensis</i>
<i>pseudomallei</i>	<i>silvatlantica</i>	<i>Burkholderia tropica</i>
<i>Burkholderia</i>	<i>Burkholderia</i>	<i>Burkholderia tuberum</i>
<i>pseudomultivorans</i>	<i>solanacearum</i>	<i>Burkholderia</i>
<i>Burkholderia</i>	<i>Burkholderia soli</i>	<i>ubonensis</i>
<i>pyrocinia</i>	<i>Burkholderia</i>	<i>Burkholderia udeis</i>
<i>Burkholderia</i>	<i>sordidicola</i>	<i>Burkholderia unamae</i>
<i>rhizoxinica</i>	<i>Burkholderia</i>	<i>Burkholderia vandii</i>
<i>Burkholderia</i>	<i>sprentiae</i>	<i>Burkholderia</i>
<i>rhynchosiae</i>	<i>Burkholderia stabilis</i>	<i>vietnamiensis</i>
<i>Burkholderia sabiae</i>	<i>Burkholderia</i>	<i>Burkholderia</i>
<i>Burkholderia sacchari</i>	<i>symbiotica</i>	<i>xenovorans</i>
<i>Burkholderia sartisoli</i>	<i>Burkholderia telluris</i>	<i>Burkholderia</i>
<i>Burkholderia</i>	<i>Burkholderia terrae</i>	<i>zhejiangensis</i>
<i>sediminicola</i>	<i>Burkholderia terrestris</i>	

*Burkholderia pseudomallei*: The human pathogen *B. pseudomallei* is a motile gram-negative soil bacterium which is principally an environmental saprophyte. *B. pseudomallei* causes melioidosis, an infection that is endemic in tropical areas of Northern Australia (where it originated) and Southeast Asia (where it has since migrated to), and occurs sporadically in many other countries. Like *B. pseudomallei*, the closely related *B. thailandensis* and *B. oklahomensis* also live in soil, but are much less pathogenic and are phylogenetically distinct from *B. pseudomallei* and *B. mallei*.

*Burkholderia mallei*: A monophyletic group of isolates within the *B. pseudomallei* group that migrated to Southeast Asia has diverged to become the mostly equine pathogen species *B. mallei*, which can cause the severe rapidly life-threatening pneumonic infections of domestic equids and humans known as glanders. Epidemiologic and molecular data collected from *B. mallei* isolates from equines in Punjab, Pakistan from 1999 through 2007 show that recent outbreaks there are genetically distinct from available whole genome sequences from elsewhere in the world, and that these new genotypes are persistent and ubiquitous in Punjab, probably due to human-mediated movement of equines (47). *B. mallei*, unlike *B. pseudomallei*, do not survive well in soil.

Other *Burkholderia* species: Other members of the *Burkholderia* genus are also human pathogens. Members of the *B. cepacia* complex (Bcc), including the bacterium *B. cenocepacia*, are opportunistic human pathogens associated with lung infections in people with cystic fibrosis (CF) and certain other immune-compromised conditions. *B. cepacia* complex is a group of phenotypically similar non-fermenting, aerobic, gram-negative rods that infect 2 to 8% of patients with cystic fibrosis (CF). Bcc comprises several distinct species of *Burkholderia*.

*Burkholderia thailandensis* (10, 37, 56, 81) and *Burkholderia oklahomensis* (37, 38) are two species that are very genetically close to *B. pseudomallei*. These two species are attenuated when compared to *B. pseudomallei* but are genetically close enough as to cause potential problems with the confirmation of *B. pseudomallei*.

## Virulence Factors.

Little is known conclusively about the virulence factors of *B. mallei* and *B. pseudomallei*. Some candidate virulence factors of *B. pseudomallei* have been identified in the literature, but little conclusive information is available, especially in human virulence. The virulence factors associated with *B. mallei* probably include many of the same elements as *B. pseudomallei* (34, 41, 63, 72, 87, 92, 96). Incidental environmental exposure to *B. pseudomallei*, as seen in Australia and Thailand, does not often result in disease. Of the individuals that do develop disease, 50-70% of them have a predisposing medical condition, such as diabetes, alcoholism, cirrhosis, renal disease, thalassemia, cystic fibrosis, or they are using immunosuppressive drugs (e.g., corticosteroids). Melioidosis may be an asymptomatic disease and remain inactive for long periods. Some asymptomatic disease cases may activate up to 30 years after exposure, often associated with in conjunction with an immune-compromising state (15).

Capsule: A 200-kd group 3 capsular polysaccharide composed of a homopolymer of -3)-2-O-acetyl-6-deoxy-β-D-manno-heptopyranose-(1-.1 (41). The capsule may contribute to survival in serum by reducing complement factor C3b deposition. Capsule mutants are highly attenuated in hamsters and mice.

Exotoxins: There have been several reports in the literature about *B. pseudomallei* exotoxins, but the genes encoding these exotoxins have not been identified and no defined exotoxin mutants have been constructed. The role of exotoxins as *B. pseudomallei* virulence factors is highly controversial, and there appears to be no correlation between in-vitro cytotoxicity and in-vivo virulence.

Endotoxin: *B. pseudomallei* lipopolysaccharide (LPS) contains lipid A composed of amide-linked 3-hydroxyhexadecanoic acids, that is often considered an endotoxin. *B. pseudomallei* LPS is longer than the fatty acid chains of enterobacterial LPS. This translated to 10-100 times weaker endotoxic activity than enterobacterial LPS in rabbits, lethal toxicity in GalN-sensitized mice, and macrophage activation assays. However, the mitogenic activity (ability to trigger cell division) was much higher than enterobacterial LPS (92, 99).

LPS O-antigen: The outer components of LPS are termed the O-antigen, and in *B. pseudomallei* it is an unbranched heteropolymer with repeating D-glucose and L-talose units, but the *B. mallei* O-antigen has substitutions in the L-talose units (34). Mutations in the O-antigen result in attenuation in hamsters, guinea pigs, and infant diabetic rats (92).

Type III secretion system (T3SS): *B. pseudomallei* contain three distinct T3SS loci: T3SS1, T3SS2, and T3SS3. T3SS1 and T3SS2 are similar to the plant pathogen *Ralstonia solanacearum* T3SS genes and are not necessary for virulence in hamsters. The T3SS3 locus, also seen in *B. mallei*, is similar to *Salmonella* and *Shigella* TTSS and is required for full virulence in both mice and hamsters (89).

Type II secretion system (T2SS): T2SS is required for the secretion of several exoproteins, including protease, lipase, and phospholipase C. Those products appear to play a minor role in *B. pseudomallei* pathogenesis (29).

Quorum sensing: *B. pseudomallei* encode three *luxI* homologues that produce at least three quorum-sensing molecules and five *luxR* homologues to sense these signals. Mutations in all any of the genes result in strains with decreased virulence in hamsters and mice (90, 91).

Flagella: The primary role of flagella is to provide bacteria with locomotion but they also often has function as a sensory organelles. Flagellin is the globular protein that forms the principle structure to bacterial flagella. Flagellin mutants are attenuated in mice, but not in hamsters or infant diabetic rats (typical animal models used). Passive exposure studies demonstrated that flagellin-specific antiserum was capable of protecting infant diabetic rats from challenge with *B. pseudomallei* (9, 27, 34).

Pili/fimbriae: Pili are protein hairlike appendages found on the surface of many bacteria that act as various receptors. The Type IV pili aid in bacterial motility and *B. pseudomallei* possess 4-8 type IV pilin gene clusters. A *pilA* mutation resulted in decreased attachment to cultured respiratory cell lines. The *pilA* mutant was not attenuated in intraperitoneal challenged mice, but was slightly attenuated by the intranasal challenge route (32).

## Epidemiology and Endemic Areas.

*B. pseudomallei* (melioidosis) is widely distributed in water and soil in many tropical and subtropical regions. Melioidosis is endemic in Southeast Asia and northern Australia. *B. pseudomallei* has been determined to have come solely out of Australia and expanded into Southeast Asia over time (67). It is most prevalent during the rainy season in people who have direct contact with wet soils and who have predisposing medical conditions (e.g., diabetes mellitus). In northeastern Thailand, *B. pseudomallei* has accounted for 20% of community-acquired septicemia cases and the organism can be isolated from 50% of rice paddies. Melioidosis can reactivate years after primary infection and result in chronic and life-threatening disease, often confusing epidemiological investigations (15, 22, 59). After the US engagement in Vietnam, the disease was termed "the Vietnamese time bomb" by some (15, 49). An interesting area of spread is into Brazil in South America (74).

*B. mallei* (glanders) is still infrequently reported in northern Africa, the Middle East, South America, and Eastern Europe. The horse is considered the reservoir of *B. mallei*. Glanders is transmitted directly by bacterial invasion of the nasal, oral, and conjunctival mucous membranes, inhalation, and by invasion of abraded or lacerated skin. *B. mallei* may survive in stable bedding, manure, feed and water troughs, wastewater, and enclosed equine transporters. Transmission from handling contaminated fomites, such as grooming tools, hoof-trimming equipment, harnesses, tack, feeding equipment, and veterinary equipment, has been documented.

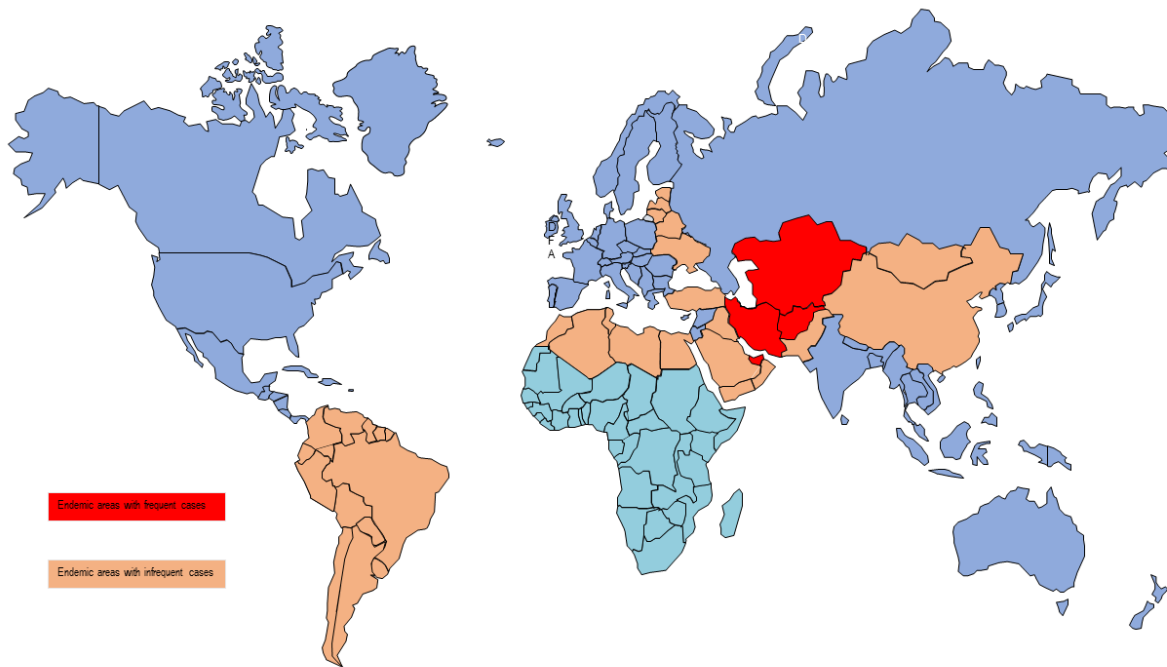


Figure 37. *Burkholderia mallei* endemic areas

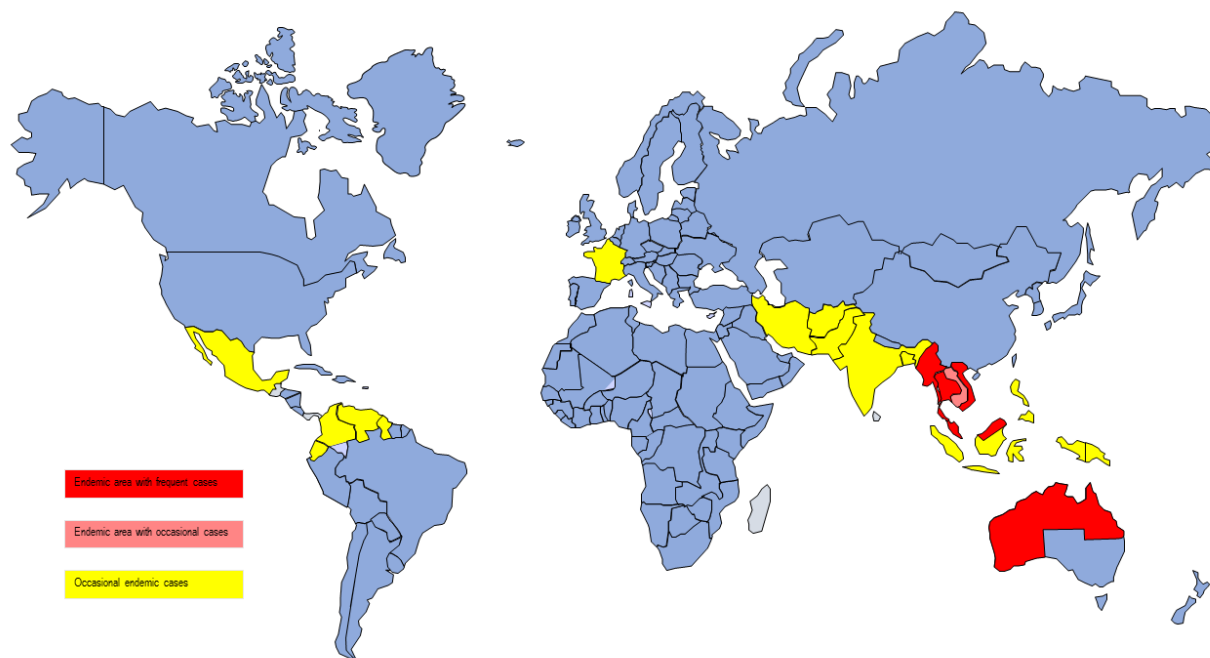


Figure 38. *Burkholderia pseudomallei* endemic areas

## LABORATORY DIAGNOSTICS

### Biosafety Information.

Biosafety in Microbiological and Biomedical Laboratories (BMBL) Containment Recommendations (5th Edition): Primary isolations from patient fluids or tissues may be performed with biosafety level (BSL)-2 practices, containment equipment, and facilities in a BSC. Procedures must be performed under BSL-3 containment whenever infectious aerosols or droplets are generated, such as during centrifugation or handling infected animals, or when large quantities of the agent are produced.

## DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

Procedures conducted outside of a BSC (centrifugation, animal manipulation, etc.) that generate infectious aerosols require respiratory protection. Sealed cups should be used with all centrifuges and these should be opened only inside a BSC. Gloves should be worn when working with potentially infectious material or animals. Animal work with *B. mallei* should be done with animal BSL-3 practices, containment equipment, and facilities.

## Diagnostic Information.

## General.

*Burkholderia* spp. are gram-negative, motile, encapsulated, non-spore-forming, aerobic, rod-shaped bacteria with a width of 0.4-0.8  $\mu\text{m}$  and a length of 2-5  $\mu\text{m}$ . They causes the diseases "melidosis" or "glanders", the former being an environmental organisms with incidental human disease and the later which is principally a zoonotic disease with human infections being incidental. While the two organisms are similar in many respects, testing for *B. pseudomallei* (melidosis) and *B. mallei* (glanders) represent testing for two separate diseases. The similarity of the organisms to each other and other *Burkholderia* spp. present a challenge for diagnostics that are specific for each agent.

## Staining

Gram stain reveals small irregularly staining, gram-negative, bacilli. Organisms from older cultures may be pleomorphic.

Wright stain: Wright's stain may reveal bipolar "safety pin" staining.

Methylene blue: Methylene blue stain may reveal bipolar "safety pin" staining.

## Metabolic Information:

The organisms can be cultured and identified from abscesses / wounds, secretions, sputum, and sometimes blood and urine with standard bacteriological medium; adding 1-5% glucose, 5% glycerol, or meat infusion nutrient agar may accelerate growth. Primary isolation requires 48-72 h in agar at 37°C, although the optimal temperature has been reported to be 40°C. Automated blood culture methods are typically more rapid. *B. pseudomallei* is generally more rapidly growing and less fastidious than *B. mallei* which typically take up to 72 hrs to grow (70).

Selective inhibition of *B. mallei*, *B. pseudomallei* and *Pseudomonas aeruginosa* by growth conditions:

- *B. mallei* does not grow at 42 °C; *B. pseudomallei* and *P. aeruginosa* do.
- *B. mallei* grow at 21°C; *P. aeruginosa* does.
- *B. mallei* does not grow in 2% sodium chloride solution, nor on MacConkey agar; both *B. pseudomallei* and *P. aeruginosa* do.

## Optimal temperature: 35-37°C

*B. pseudomallei*

- Upper temperature: 42°C
- Lower temperature: 25°C

*B. mallei*

- Upper temperature: 41°C
- Lower temperature: 21°C

## General Culturing Information

General: Cultures on blood agar often become non-viable after 3-4 days and survival at refrigerated temperatures is poor.

## Nutrient agar (NA).

*B. pseudomallei*

*B. mallei*: Growth is typically slow on nutrient agar, but is rapid (2 days) when enhanced with 1% to 5% glucose and/or glycerol, and on most meat infusion nutrient media. Colonies are typically opaque to grayish-white. *B. mallei* colonies typically are about 1 mm in width, white (turning yellow with age).

## Sheep (horse) blood agar (SBA).

*B. pseudomallei*: Colonies are non-hemolytic and cultures typically become positive in 24-48 h (this rapid growth rate differentiates the organism from *B. mallei*, which typically takes a minimum of 48 h to grow).

*B. mallei*: Colonies are non-hemolytic, typically about 1 mm in width, white (turning yellow with age), and semitranslucent and viscid and typically takes a minimum of 48-72 h to grow.

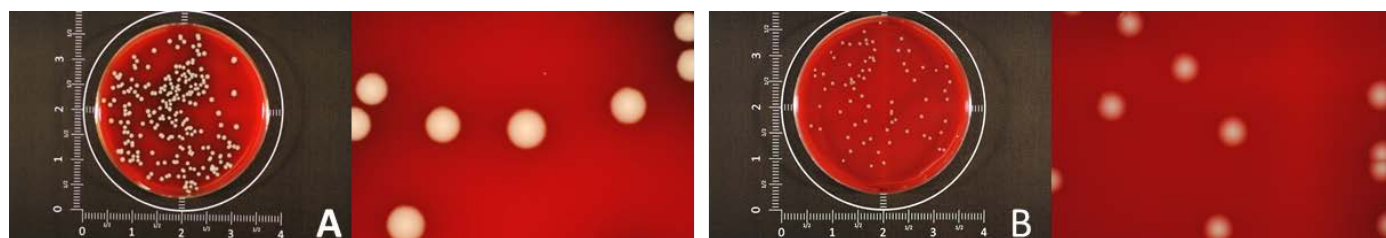


Figure 39. A) *B. pseudomallei* on SBA at 48h. B) *B. mallei* on SBA at 48 h. *B. mallei* does not grow as well at 48h as does *B. pseudomallei*.

MacConkey agar (Mac). *B. pseudomallei* grows well but *B. mallei* does not. They do not grow on Salmonella-Shigella agar (70).



Figure 40. *B. pseudomallei* on MacConkey agar at 48 h. Note wrinkled colony morphology.

Chocolate agar (CA):

*B. pseudomallei*: Cultures typically become positive in 24-48 h and usually (but not always) display the wrinkled colony morphology.

*B. mallei*: Colonies typically about 1 mm in width, white, semitranslucent, viscid, and typically take a minimum of 48-72 h to grow.

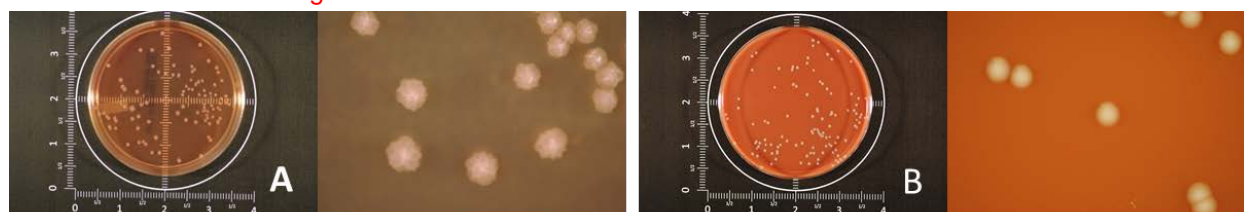


Figure 41. A) *B. pseudomallei* on CA at 48h. B) *B. mallei* on CA at 48 h. *B. mallei* does not grow as well at 48h as does *B. pseudomallei*.

Glycerin-potato medium.

*B. mallei*: Colonies have a clear honey-like layer by day three, later darkening to brown or reddish-brown.

*B. pseudomallei*: (not defined in the literature).

Blood culture media. Blood cultures for *B. mallei* are rarely positive upon presentation unless the patient is moribund. In contrast, blood cultures for *B. pseudomallei* septicemia are often positive and urine culture may be positive, especially if prostate inflammation or renal abscesses are present.

#### Selective Culturing Information

Ashdown's selective agar (ASA): *B. pseudomallei* grows well on Ashdown's medium but *B. mallei* does not. Ashdown's medium contains gentamicin and provides selective culture from nonsterile sites or where contaminants can be problematic. Ashdown's selective agar is the currently favored medium for the isolation and presumptive identification of *B. pseudomallei* in areas where melioidosis is endemic (6).



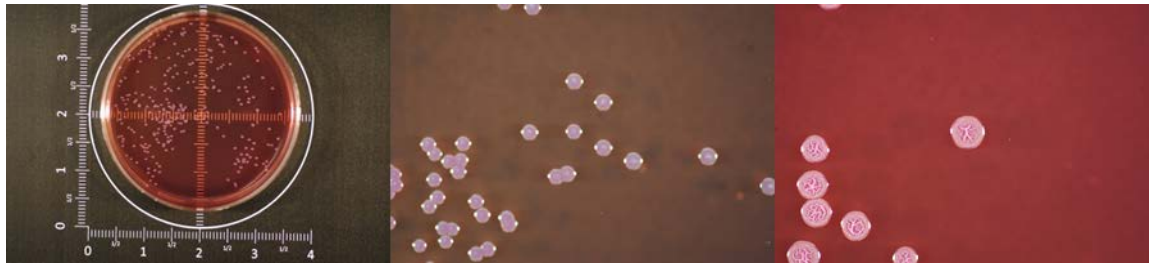


Figure 42 *B. pseudomallei* on Ashdowns agar at 48h. Colonies can be smooth or rough at 48h.

At least two distinct colony phenotypes are commonly observed on this medium, due to the differential uptake of crystal violet and neutral red or the differential production of ammonia and oxalic acid. Most strains appear lavender after 2 to 3 days of incubation at 37°C, but some isolates appear deep purple. After 5 days at 37°C, the colonies often become dull and wrinkled and emit a distinctive sweet earthy smell (7, 23, 48, 66, 97).

*Burkholderia pseudomallei* selective agar (BPSA): BPSA improves recovery of the more easily inhibited strains of *B. pseudomallei*. BPSA was more inhibitory to *P. aeruginosa* and *B. cepacia* and should make recognition of *Burkholderia* species easier due to distinctive colony morphology. BPSA also inhibited *Enterococcus*, *Escherichia*, *Staphylococcus*, and *Streptococcus*. These results indicate that BPSA is a potential replacement for ASA. BPSA provides large wrinkled colonies faster than ASA (48).

*Pseudomonas cepacia* (PC) agar: PC is highly sensitive and selective using crystal violet, bile salts and two antimicrobial agents as selective agents for isolation of *B. cepacia*. Some *B. cepacia* strains produce a non-fluorescent phenazine pigment, while others are non-pigmented. A sweet odor similar to that associated with *P. aeruginosa* is produced by some strains. Since Ashdown's agar is not commercially available, PC agar was evaluated for the growth of *B. pseudomallei* and *B. mallei*. Ashdown's agar was the most sensitive medium for the isolation of *B. pseudomallei* but *B. cepacia* agar was highly sensitive and selective (13-15% of other organisms tested grew) for both *B. pseudomallei* and *B. mallei*. In non-endemic areas, the use of PC agar for the isolation of *B. mallei* and *B. pseudomallei* is now recommended (39).

*B. pseudomallei*- 95% grow on this agar and change to pink, circular, white to yellow, metallic colonies.

*B. mallei* –75% grow on this agar and change to pink, circular, white to translucent, smooth, < 1 mm colonies.

*B. mallei*-selective media: There is a *B. mallei* selective media described in the literature (94)

OFPBL Agar: OFPBL is OF (oxidation-fermentation) basal medium supplemented with polymyxin B, bacitracin, lactose and agar. The indicator, bromthymol blue, aids in the detection of *B. cepacia* isolates through a color change in the medium. These investigators reported isolating *B. cepacia* on OFPBL agar but some *Burkholderia* spp. do not grow on it (45).

*B. pseudomallei*- 90% grow on this agar and change color to yellow, circular, smooth, convex, entire, and yellow to translucent colonies.

*B. mallei* – only 55% grow on this agar and change color to yellow or no change, circular, pinpoint, translucent colonies.

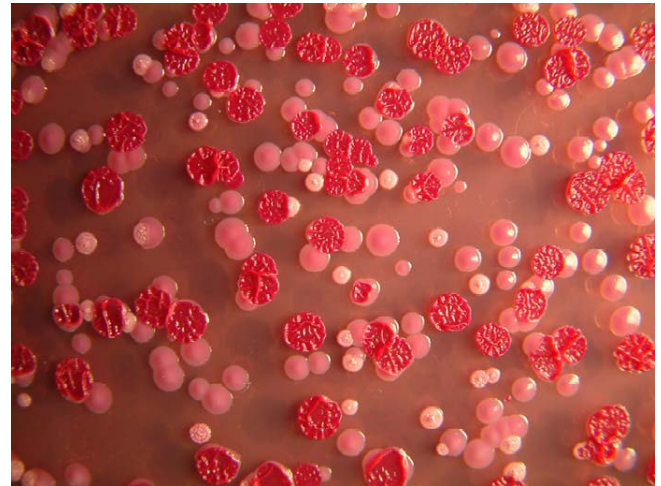


Figure 43. *B. pseudomallei* on Ashdowns agar at 96h. Note the mixed culture morphologies present; a natural characteristic of *B. pseudomallei*.

### Diagnostic Tests.

General.

*B. mallei* is an oxidase-variable, small, non-motile, non-sporulating, non-encapsulated aerobic gram-negative bacillus approximately 2 to 4 µm long and 0.5 to 1 µm wide. *B. mallei* is facultative anaerobic in the presence of nitrate. Size may vary by strain and by environmental factors, including temperature, growth medium, and age of culture. Organisms from young cultures and fresh exudate or tissue samples typically stain in a bipolar fashion with Wright stain and methylene blue. Organisms from older cultures may be pleomorphic.

*B. pseudomallei* is an oxidase-positive, small, motile, non-sporulating, non-encapsulated aerobic gram-negative bacillus approximately 2 to 5 µm long and 0.4 to 0.8 µm wide. *B. pseudomallei* is intrinsically resistant to aminoglycosides and polymyxins. This unusual antibiotic profile (gentamicin and colistin resistance, but amoxicillin-clavulanate susceptibility) in an oxidase-positive, gram-negative bacillus is helpful for identifying *B. pseudomallei*.

A recently published paper from Australia proposed a highly sensitive *B. pseudomallei* identification algorithm that makes use of screening tests (Gram-stain, oxidase test, gentamicin, and polymyxin susceptibility testing) combined with monoclonal antibody agglutination testing and gas-liquid chromatography analysis of bacterial fatty acid methyl esters.

**Biochemical Identification:** To distinguish *B. pseudomallei* from *B. cepacia* and *B. mallei*, the determination of arginine dihydrolase (ADH), lysine decarboxylase (LDC), ortho-nitrophenyl-gamma-D-galactopyranosidase (ONPG) and the reduction from nitrate to nitrite (NIT) can be used. *B. pseudomallei* is ADH and NIT-positive and LDC and ONPG-negative, whereas *B. cepacia* is ADH and NIT-negative but LDC and ONPG-positive. *B. thailandensis* strains assimilate arabinose whereas *B. pseudomallei* is not able to assimilate arabinose (54, 83). The use of the commercial API20NE was able to correctly identify 97.5% of 400 Australian clinical strains based on the biochemical reactions and colony characteristics, Gram's stain, oxidase reaction, colistin and gentamicin resistance (26), but API20NE alone was only 37% effective (54).

	<i>B. pseudomallei</i>	<i>B. mallei</i>	<i>B. cepacia</i>	<i>B. thailandensis</i>
Oxidase	+	V	+	+
Growth at 42°C	+	—	V(43%)	+
Nitrate reduction	+	—	—	+
Nitrate - gas	+	+	—	+
Arginine dihydrolase	+	+	—	+

Lysine decarboxylase	—	unkn	+	unkn
Ortho-nitrophenyl-gamma-d-galactopyranosidase (ONPG)	—	—	+	—
Ribose	+	—	unkn	unkn
Erythritol	+	—	unkn	unkn
Motility	+	—		+
Arabinose	—	+ <sup>a</sup>	unkn	+
Maltose	+	—	+	+
Mannitol	+	—	unkn	+
Gentamicin <sup>b</sup>	resistant (<12mm)	sensitive (>15mm)	sensitive (>15mm)	sensitive (>15mm)

a- OIE Terrestrial Manual 2012; CHAPTER 2.5.11 GLANDERS  
b- 10-mcg gentamicin disk

#### Commercial Identification systems.

General: Commercial and automated bacterial identification systems may misidentify these organisms. In the eighth U.S. laboratory-acquired infection, such an automated system identified the agent as *Pseudomonas fluorescens* or *P. putida* (41). Commercial systems have been reported to reliably confirm the identity of *B. pseudomallei* although other investigators have reported mixed results or outright misidentifications (50, 57, 58).

Fatty acid methyl ester (FAME) analysis: *B. pseudomallei*, can be distinguished from the closely related but attenuated *B. thailandensis* by gas chromatography (GC) analysis of fatty acid derivatives. A 2-hydroxymyristic acid derivative (14:0 2OH) was present in 95% of *B. pseudomallei* isolates and no *B. thailandensis* isolates. No significant differences were observed in fatty acids between strains of human and environmental origins (53, 54, 56, 69).

Vitek: The Vitek 1 (bioMérieux) was found to be highly sensitive, having identified 99% of the 103 *B. pseudomallei* isolates tested but the Vitek 2 identified only 19% of these same isolates until the software was updated. The failure of the Vitek 2 to correctly identify *B. pseudomallei* was largely due to differences in the biochemical reactions achieved compared to expected values in the database. It was suggested that this deficiency in the Vitek 2 may be due to the large number of uncertain results reported for these isolates misidentifications (50, 57, 58). False positive *B. pseudomallei* results for *B. thailandensis* and other incorrect species calls are due to the Vitek database only having 4 species available for matches. Caution in the interpretation of Vitek on *Burkholderia* should be practiced.

Biolog: There are no significant articles on the use of Biolog GN2 or GENIII for the identification of *Burkholderia* spp. The use of the Biolog phenotype microarray (PM) system has been reported, however. They used the PM-1, PM-2, and PM-3 microtiter plates for sources of carbon, nitrogen, and phosphorus in a *B. thailandensis* quorum-sensing study (11). In internal studies, using the GENIII system, from a set of 136 assays, *B. pseudomallei* were reported as *B. ambifaria* (15%), *B. oklahomensis* (3%), *B. pseudomallei* (20%), *B. thailandensis* A or B (17%), or 'no identification' (25%). Using the GENIII system, internal data only exists for 8 assays involving *B. mallei* and they were reported as *B. mallei* (1), *Brachymonas denitrificans* (1), *B. thailandensis* A (1), *Pseudomonas mucidolens* (1), *Ralstonia pickettii* (1), *Simplicispira metamorpha* (1) or 'no identification' (2).

#### Immunoassay Tests.

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology). The similarity of *Burkholderia* organisms present a big problem for immunoassay based systems due to similar antigenic proteins between the species. Again, while the two organisms are similar, testing for *B. pseudomallei* (meliodosis) and *B. mallei* (glanders) represent testing for two separate diseases and the immunoassay information separates the information as best possible. Particular to immunoassays for glanders are the assays directed at animals.

#### Direct agent immunoassays.

*B. pseudomallei* (meliodosis)

Direct fluorescent antibody (DFA): In one study, 776 specimens from patients with suspected melioidosis were tested by IF and the sensitivity was reported as 66% with a specificity of 99.5-99.4%. In that study 4 of 622 specimens were culture negative for *B. pseudomallei* yet positive by an IF method; 4 specimens were reported as false positives; 2 urine samples that grew *Pseudomonas aeruginosa* and *Acinetobacter* spp. and 2 respiratory secretions that grew mixed respiratory flora (93, 98).

Enzyme-linked immunosorbent assay (ELISA): ELISAs for direct detection of *B. pseudomallei* have been developed but are not widely available outside of a few regional uses. In one report, a specific monoclonal antibody in a sandwich ELISA is used to capture a *B. pseudomallei* antigen directly from clinical specimens that were culture positive for *B. pseudomallei* were shown to be 75% sensitivity and 98 % specificity (4). Others have used monoclonal antibodies to detect *B. pseudomallei* as well as prepare affinity-purified antigens for use in an indirect ELISA for acute septicemic melioidosis cases (75). In a *B. pseudomallei* urinary antigen assay, the assay found 96% of 89 patients with septicemic melioidosis, all six patients with urinary tract infection, and 80% of 40 patients with other *B. pseudomallei* localized infections. The urine ELISA negative in a healthy population but urine from hospitalized patients with diagnoses other than melioidosis gave a positive result (28).

Latex agglutination assays: Monoclonal antibody-based latex agglutination tests have been used for the rapid identification of *B. pseudomallei* in blood cultures (5, 30, 76, 84). Latex agglutination has also been used to detect *B. pseudomallei* from concentrated patient urine (80).

Serological testing. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for all serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

Enzyme-linked immunosorbent assay (ELISA):

For *B. pseudomallei* serodiagnosis, an ELISA for antibodies to a partly purified culture filtrate antigen was developed. The immunoglobulin G ELISA exhibited a sensitivity of 96% and a specificity of 94%, whereas the immunoglobulin M ELISA had a sensitivity of 74% and a specificity of 99% (18). In a different study using sera from patients with proven or suspected *B. pseudomallei* infection, the immunoglobulin G was detectable in 94-100%, immunoglobulin A was detected in 86-100%, and immunoglobulin M in 66-85%, whereas immunoglobulin E was not detected (17). Another ELISA, a purified glycolipid antigen from *B. pseudomallei* was used and the authors reported immunoglobulin G detection in 49 of 50 sera resulting in a sensitivity of 98.0% and specificity of 98.9% (68). Other studies, based on a lipopolysaccharide-specific antibodies, demonstrated a sensitivities between 62 and 96.0% and specificities approaching 100% (1, 85). An ELISA against affinity-purified antigen demonstrated a sensitivity of 82% and a specificity of 72% (13). One author suggested that an internationally standardized serodiagnostic test for melioidosis is needed (27).

*B. mallei* (glanders): Serological tests for glanders in humans are not often consistent nor are they particularly timely for rapid response; serological response typically requires 7-10 days and sometimes up to 3 weeks for appropriate antibody responses to develop. Despite shortcomings, serology is being used routinely in veterinary medicine (e.g., in horses, goats, and dairy cows) (19, 83). Serodignositc ELISAs for human glanders have been developed (41, 65, 71). One ELISA could differentiate serum from a glanders' patient from sera from patients with clinical cases of anthrax, brucellosis, tularemia, Q fever, and spotted fever, but could not distinguish glanders from melioidosis (41). The major problem with most immunoassays is antigen being used. Another ELISA developed with two recombinant proteins, however, exhibited 100% sensitivity and specificity for glanders diagnosis with human sera (65).

Complement fixation test (CFT): CFT are more specific but less sensitive and may require 40 days for conversion. CFT tests are considered positive if the titer is equal to, or exceeds 1:20 but CFT may not detect chronic cases of glanders. CFT assays have been reported to be 90-95% sensitive but false negatives and false postives have been reported (20, 21, 62, 77).

Hemagglutination test (HAI or IHAT): Although sensitive, high background titers (up to 1:320) in agglutinin tests may make the test difficult to interpret when titers from 0 to 320 may be significant (35, 41).

Mallein testing: Mallein testing of animals is quick and cheap. It is based on an allergic hypersensitivity reaction where mallein, a protein fraction of *B. mallei*. Mallein can be injected intradermally, subcutaneously, or applied as an eye-drop. If an animal has glanders, it will show as swelling around 48 hours post application. Mallein proteins are not well standardized world wide causing some variability in testing. Cross-reactions have also been reported with *Streptococcus equi* infections, resulting in false positive mallein reactions (43, 61). A human mallein skin test was attempted, but the

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normal serological delay of several weeks postinfection for positive antibody rendered it of little diagnostic value. The human test consists of 0.1 mL of 1:10,000 diluted commercial mallein injected intradermally into the forearm with observations at 24 and 48 h. Five of seven patients tested positive as early as the 18<sup>th</sup> day of the disease (73).

Rose Bengal plate agglutination test (animals only): Inconsistencies with the mallein test spurred development of a different on-site screening test for glanders. The test has been reported to be more sensitive than the mallein test and is simple and rapid to perform (61).

### Nucleic Acid Detection Tests.

The genomes of *B. pseudomallei* and *B. mallei* are sufficiently unique that they deserve some specific information. The genome of *B. pseudomallei* consists of two chromosomes of approximately 4 Mb and 3 Mb each (46). Chromosome 1 encodes predominately the core housekeeping genes and chromosome 2 encodes predominately the accessory genes. The genes on chromosome 2 contain plasmid-like replication and accessory genes, suggesting it may have been derived from a plasmid that became an indispensable. There are "genomic islands", insertion sequences, prophages, and integrated plasmids (33).

The genome of *B. mallei* is 99% identical in conserved genes but has about 1.4 Mb less DNA than *B. pseudomallei*. *B. mallei* evolved from a strain of *B. pseudomallei* after the latter had infected an animal and over time shed unneeded genes responsible for survival in a soil environment. Thus *B. mallei* is now considered a human/animal maintained organism and not an environmental one. The genome is about 3.5 Mb with a 2.3 Mb "megaplasmid." Many insertion sequences and phase-variable genes are still present from its derivation from *B. pseudomallei*. The genome is still partitioned like *B. pseudomallei* where chromosome 1 has genes relating to capsule formation, metabolism, and lipopolysaccharide biosynthesis and chromosome 2 has most of the virulence-associated and secretion systems genes (33).

The similarity of *Burkholderia* organisms also presents problem for nucleic acid detection systems, similar to the problem with immunoassays. Again, while the two organisms are similar, testing for *B. pseudomallei* (meliodosis) and *B. mallei* (glanders) represent testing for two separate diseases and the nucleic acid information separates the information as best possible.

Polymerase chain reaction-based techniques (PCR):

- PCR procedures have been developed to identify *B. mallei* and *B. pseudomallei* (31, 44, 55, 64, 78, 86, 88, 95).
- A multiplex PCR, that consists of primers that flank a 10-bp repetitive element in *B. pseudomallei* and *B. mallei*, amplifies PCR fragments of varying sizes between (400–700 bp in *B. pseudomallei* and *B. mallei*; a 308 bp metalloprotease gene in *B. thailandensis*; a 245 bp fragment in *B. pseudomallei* and *B. thailandensis* (55).
- The 115-base-pair region within orf2 of the *B. pseudomallei* type III secretion system gene cluster was used to distinguish *B. pseudomallei* from other microbial species (60, 64).

Joint Biological Agent Identification and Diagnostic System (JBAIDS): This *Burkholderia* spp. (Glanders) detection kit, when used with the JBAIDS platform (hardware and software), is capable of detecting *B. mallei* and *B. pseudomallei* organism. Specifically, this *Burkholderia* spp. test is based on a PCR assay that recognizes DNA target sequences present in *B. mallei* and *B. pseudomallei*. For optimal assay performance, the DNA of the pathogen has to be initially isolated from specimens using appropriate sample purification kits and procedures. This PCR-based test, however, does not distinguish between live or dead organisms.

### Other Diagnostic Methods.

None identified.

### Characterization.

Most of the current techniques for characterization have been applied to *Burkholderia* spp. While all systems are able to differentiate species and characterize strains, the concordance to epidemiological information or other pertinent information is often tenuous at best (3).

16S rRNA sequencing: 16S ribosomal RNA gene-sequence analysis identified *B. mallei* from other *Burkholderia* species in the 2000 US laboratory-acquired infection (36, 41).

Randomly amplified polymorphic DNA (RAPD): RAPD has been applied to studies of *B. pseudomallei* and *B. mallei*. In the *B. pseudomallei* study, RAPD, using a five primer set, was valuable in

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strain association with the epidemiology (42). In a collection of 14 isolates of *B. mallei* that were characterized, the RAPD analysis was better for detecting differences strains (2). RAPD has fallen into dis-use as other methods are developed.

Pulsed-field gel electrophoresis (PFGE): PFGE has been used to identify and differentiate strains of *B. pseudomallei* in outbreaks. PFGE may be useful for identification and characterization of *B. mallei*, although these methods may be more labor intensive and time consuming than gene sequencing (12, 14, 16, 51).

Ribotyping: Ribotyping (42, 79), including the use of an the automated ribotyping system(52), has been to study *B. pseudomallei* in Austrilia and in Thialand. *Bam*HI was used initially but in the automated system *Eco*RI was more discriminating.

repPCR: In one study, repPCR was able to discriminate species of *Burkholderia* including *B. mallei* and *B. pseudomallei*. repPCR data showed some concordance with previously determined typing results for *Burkholderia*. In another study, BOX-PCR typing compared favorably with MLST and PFGE performed on the same isolates, discriminating the majority isolates showing relatedness between epidemiologically linked isolates (24).

Multilocus sequence typing (MLST): MLST is by far the most common typing method for *Burkholderia* species (2, 82), including *B. pseudomallei* and *B. cepacia*. For *B. pseudomallei*, seven genes are used; *ace*, *gltB*, *gmhD*, *lepA*, *lipA*, *narK*, and *ndh* (40). For *B. cepacia*, the seven genes differ slightly; *atpD*, *gltB*, *gyrB*, *recA*, *lepA*, *phaC*, and *trpB* (8). There are two public web based resources for use of MLST. MLST of *B. mallei* has been done but RAPD analysis was the better method used for detecting differences those strains (2).

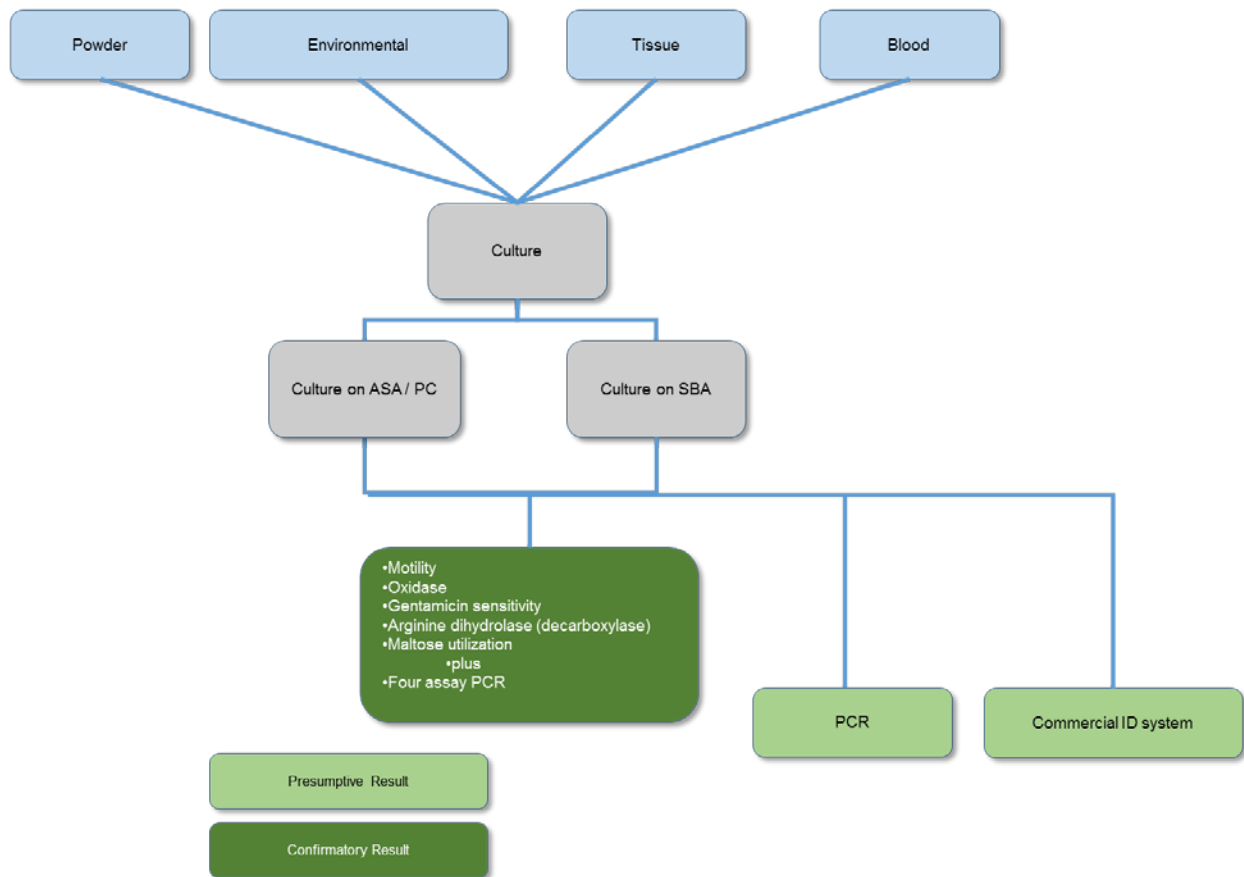
Multilocus variable number tandem repeat analysis (MLVA): A simplified 4-locus (MLVA-4) for rapid typing and compared results with PFGE and MLST for a large number *B. pseudomallei* isolates; it discriminated between 65 multilocus sequence types and showed relatedness between epidemiologically linked isolates from outbreak clusters and between isolates from individual patients. MLVA-4 can establish or refute that a clonal outbreak of melioidosis has occurred within 8 h of receipt of bacterial strains (25).

## Confirmation.

Presumptive identification of a biological threat agent is achieved by the detection of a biological marker using a single test methodology (for example, hand-held assay [HHA]. Culture is required for confirmation testing.

Definitive identification or confirmation of *B. pseudomallei* or *B. mallei* requires a combination of techniques and assays, including most specifically, the culture of the organism to which testing can be performed directly on the organism. Culture of the organism is required on which various biochemical reactions are determined. Definitive identification or confirmation testing is performed at sanctioned reference laboratories, including reference laboratories of the CDC Laboratory Response Network (LRN). Specific LRN protocols and reagents are proprietary but any definitive identification or confirmation typically follows a general scheme.



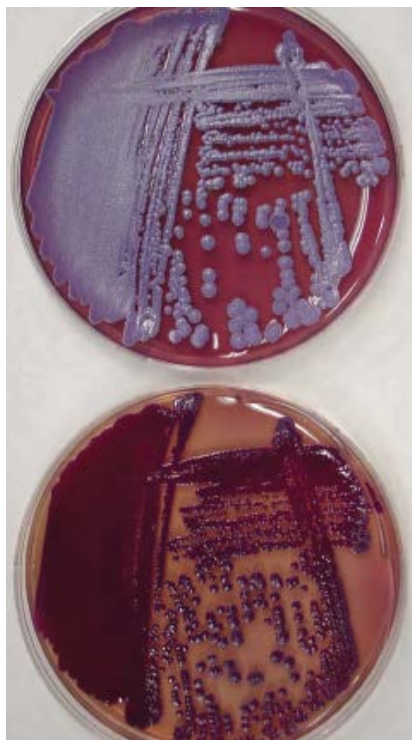




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*B. pseudomallei* colony morphologies as demonstrated on Ashdown's selective medium supplemented with 100 µg/mL streptomycin. Plates were incubated for 3 days at 37°C (a) and 5 days at 37°C (b). Photographs: Courtesy of David

Deshazer, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.



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# *Coxiella burnetii*

## OVERVIEW

Q fever is a zoonotic disease caused by *Coxiella burnetii*, which is globally distributed. Its natural reservoirs are sheep, cattle, goats, cats, some wild animals (including rodents), and ticks. The organism is shed in high numbers and transmission to humans is typically via aerosolization of infectious particles, by ingesting contaminated raw milk and cheese, and by tick vectors. The infectious dose is extremely low and a single organism may lead to human infection. Farmers, abattoir workers, and hunters are at greatest risk for exposure. *C. burnetii* is also a significant hazard in laboratory personnel who are working with the organism.

*Coxiella burnetii* was once considered an obligate intracellular parasite due to the difficulty of propagation. *C. burnetii* has unique growth requirements, including the requirement for an acidic medium (pH 4.75) and a low oxygen tension (2.5%), make routine culture difficult. Culture in eggs or cells has previously been required so routine laboratory diagnostics are not common. While highly infectious, *C. burnetii* is typically not fatal and often serology is used for diagnosis. Direct fluorescent antibody and nucleic acid assays are often used for presumptive and confirmatory diagnostics.

## HISTORY AND SIGNIFICANCE

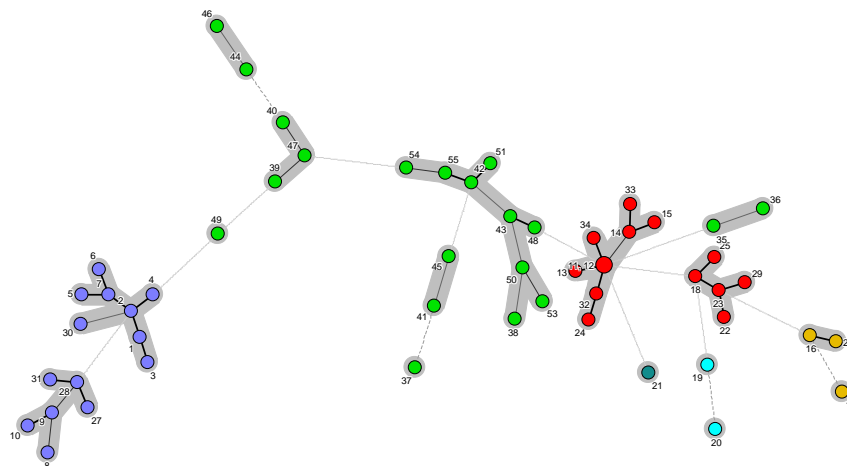
Q fever was first described in Australia by Derrick in 1935 after an outbreak of febrile illness among abattoir workers in Australia. It was called "Query fever" because the causative agent was initially unknown. Also in 1935, United States researchers isolated a rickettsia-like agent from ticks that were subsequently linked to laboratory-acquired infection, calling it Nine-Mile agent. These agents were later determined to be identical. Burnet was first to isolate and describe the organism in 1937, and Cox described vector transmission from ticks in 1938. *C. burnetii* is a "rickettsia-like" organism that is resistant to heat, desiccation, and many common disinfectants. These features allow it to survive for long periods in the environment (26). It is highly infectious by the aerosol route and humans are often quite susceptible to disease. A single inhaled organism may produce clinical illness. For all of these reasons, *C. burnetii* could be used as an incapacitating biowarfare agent.

## ORGANISM INFORMATION

### Taxonomy Information.

*Coxiella* was recognized as a genus in 1948 with *Coxiella burnetii* being the only species. Initially, *C. burnetii* were classified with the rickettsiae based on phenotypic characteristics. Based on 16S and 23S rDNA, *C. burnetii* has been moved into the order *Legionellales* that comprise the families; *Legionella*, *Aquicella*, *Rickettsiella*, and *Coxiella* (49, 62)

The original sub-classification of *C. burnetii* by restriction endonuclease digestion produced the classic six different genomic groups ((20)). There is evidence in guinea pigs that there are differences in virulence among the different genomic groups. Genomic group I isolates causing acute disease, group V causing mild to moderate disease, and no acute disease when infected with group IV and VI isolates (48). Some of the strains associated with the genomic groups include group I (Nine Mile, African, and Ohio), group IV (Priscilla and P), group V (G and S), and group VI (Dugway).



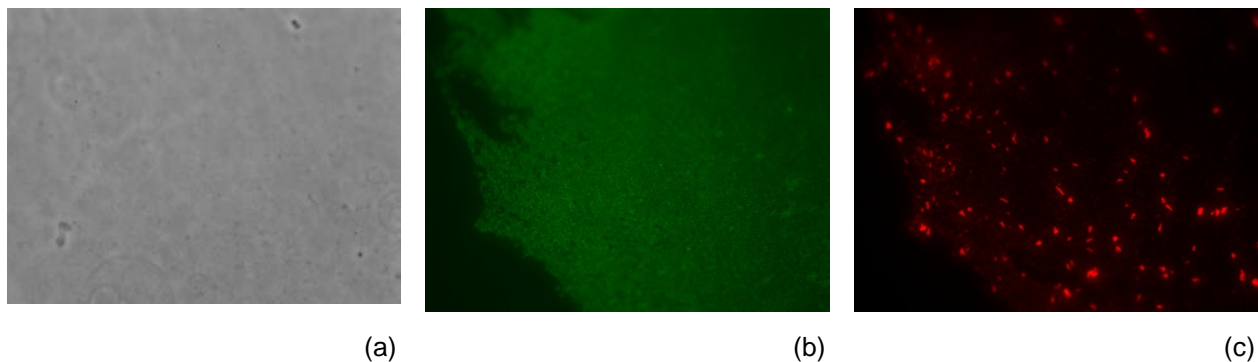
**Figure 44.** Minimum spanning tree of *C. burnetii* by multispacer sequence typing (MST) representing strain diversity (21). Node numbers are the multispacer sequence types. Green colors are additional MST types not in original genomic groups. Genomic groups (colors) based on Hendrix et al. with green colors additional MST types not in original genomic groups (20).

### Virulence Factors.

Genes encoding adhesive structures in the genome, such as pili, are absent, but there are 13 ankyrin domains (family of adaptor proteins that mediate the attachment of integral membrane proteins to a spectrin-actin based membrane skeleton) that may assist in the bacterium's attachment to its host (51).

*Coxiella* has a capsule that can function as a mechanism for motility, attachment, virulence, or protection.

**Lipopolysaccharide.** The LPS of *Coxiella* undergoes phase variation and are similar to the rough-smooth transition where the O side polysaccharide undergoes significant truncation. Phase I LPS is virulent whereas Phase II LPS is the avirulent form (39, 50, 63).



**Figure 45.** *C. burnetii* Idaho Q (COXI-004) cultured from seed stock; (a) phase contrast micrograph, (b) anti-phase II immunostain, (c) anti-phase I immunostain.

### Epidemiology and Endemic Areas.

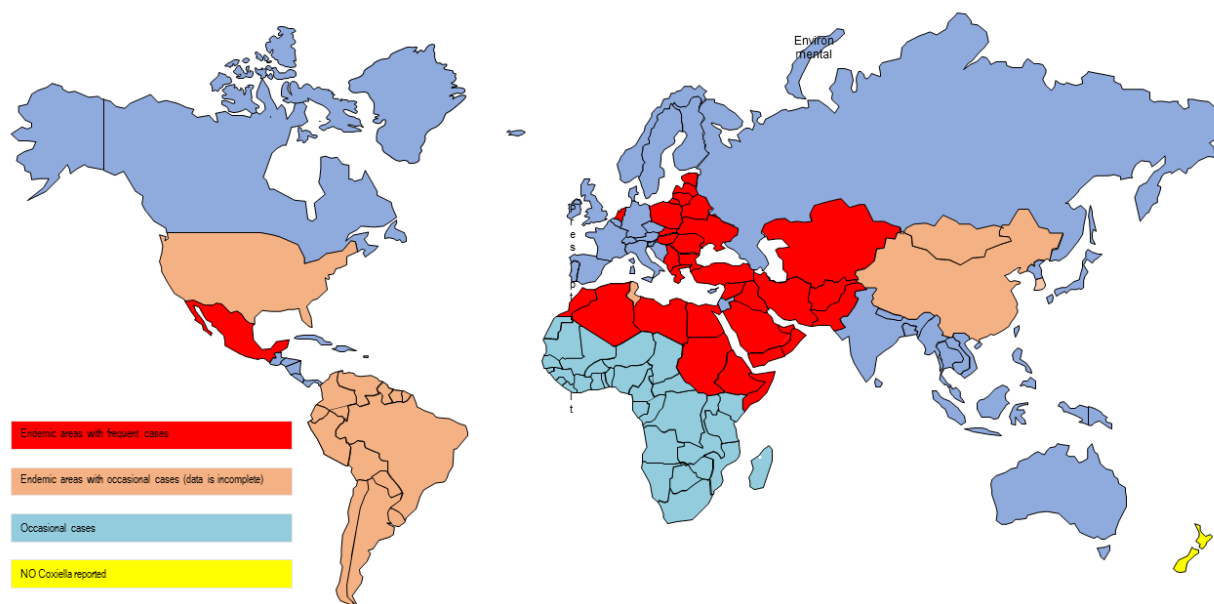


Figure 46. *Coxiella* has been reported worldwide, except for New Zealand, but data is incomplete.

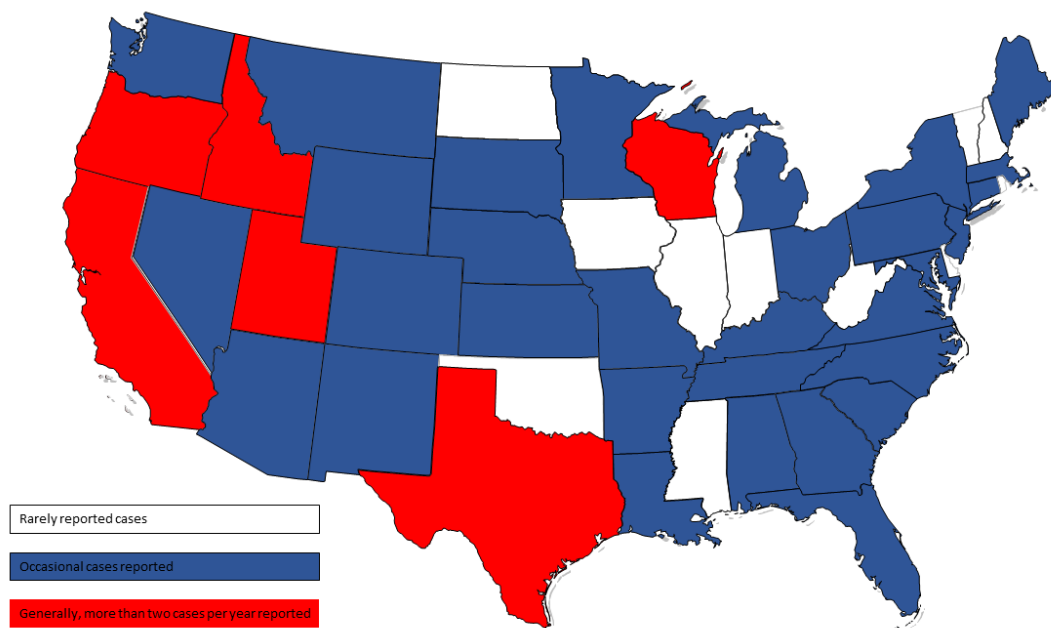


Figure 47. *Coxiella* is found in most parts of the US.

## LABORATORY DIAGNOSTICS

### Biosafety Information.

Biosafety in Microbiological and Biomedical Laboratories (BMBL) Containment Recommendations (5th Edition): Biosafety level (BSL)-2 practices and facilities are recommended for nonpropagative laboratory procedures, including serological examinations and staining of impression smears. BSL-3 practices and facilities are recommended for activities involving the inoculation, incubation, and harvesting of embryonated eggs or cell cultures, the necropsy of infected animals and the manipulation of infected tissues. Experimentally infected animals should be maintained under ABSL-3

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because infected rodents may shed the organisms in urine or feces. A specific plaque-purified clonal isolate of an avirulent (Phase II) strain (Nine Mile) may be safely handled under BSL-2 conditions.

Before performing PCR, biological samples can be inactivated, for ensuring the safety of laboratory personnel, by heating at 90°C for 30–60 min, depending of the samples' nature, their size or their weight (47).

## Diagnostic Information.

### General.

*Coxiella burnetii* is a gram-negative, non-spore forming, encapsulated, small rods to coccobacillus bacterium. *C. burnetii* exist as two distinct forms; a small cell variant (SCV) and a large cell variant (LCV). The LCV is the replicating form and the SCV akin to a sporogenic mode (10, 35). Attention must be taken in the interpretation of the results as, microscopically, *C. burnetii* can be confused with *Brucella* spp or other small gram-variable organisms. The environmental persistence of *C. burnetii* is attributable to the small cell variant (17, 35, 47).

### Staining

Gram stain: They are small gram negative coccobacilli that are normally stained with Giemsa since they stain poorly with the Gram stain. Modifications of the gram stain have been used and include the aqueous and ethyl alcohol iodine (6, 15).

Giemsa Staining: color organisms purple.

Macchiavello Staining: color organisms red.

Gimenez Staining: Gimenez is fastest stain procedurally. *C. burnetii* are characterized by a very large number of thin, pink-stained coccobacillary bacteria against a blue or green background. While they may sometimes be difficult to detect because of their size, this is compensated for by the large number of bacteria present; inclusions within the host cells appear as bright red masses against a blue or green background.

Stamp Staining: Stamp staining method is performed with 0.4% basic fuchsin solution, followed by rapid decoloration with 0.5% acetic acid solution, and counterstaining with 1% methylene blue or malachite green solution.

Modified Ziehl–Neelsen staining.

### Metabolic Information:

General: *Coxiella* are generally considered small obligate intracellular parasites requiring viable eukaryotic host cells such as laboratory animals, embryonated eggs, or tissue culture. They are acidophilic and typically metabolically inactive.

Optimal temperature: 35-37°C.

- Upper temperature: ~42°C.
- Lower temperature: ~23°C.

Optimal pH: 4.5-5.5

### General Culturing Information

Embryonated chicken egg yolk sacs have typically been the method of choice for culture. They are inoculated when the embryos are 5-7 days old. The inoculum is adjusted to kill 30% of the embryos in 5-12 days. The yolk sacs are homogenated with glass beads to free the organism.

Cell culture with chicken or mouse embryo fibroblasts, J774.16 mouse macrophages, L929 murine fibroblasts, HEL (human embryonic lung) or vero cells are more commonly being used, but tissue culture maintenance is cumbersome. The cells can be used with Eagle's minimal essential medium with 10% fetal bovine serum and isolation by plaque formation can be done if overlaid with medium, such as RPMI 1640 or Medium 199, containing glutamine and 0.8% agarose (44, 43, 55).

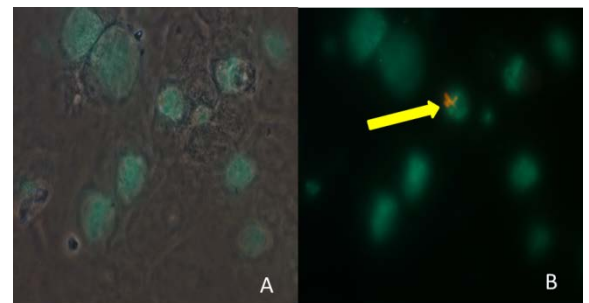


Figure 48. (A) *Coxiella* present in Vero cell culture (B) *Coxiella* phase I are red/orange and phase II are the green fluorescent stained.

Recently, *C. burnetii* were able to be cultured in cell-free media. The medium, acidified citrate cysteine medium [ACCM; citric acid/sodium citrate, L-cysteine, neopeptone, casamino acids, methyl-b-cyclodextrin, iron sulfate, RPMI w/glutamax, salts], contains specific nutrients that support *C. burnetii* metabolic activities, and is at pH 4.75 with incubation with 2.5% oxygen atmosphere. The medium purportedly mimics the acidic environment of cellular phagolysosomal compartments. Growth of various strains of *C. burnetii* differs but phase I (virulent) cells can replicate up to 1000x in 7 to 10 days (41, 40). There are differences in the ability of ACCM to support growth of different strains however (27).

Selective Culturing Information. None identified.

#### Diagnostic Tests

Laboratory Diagnosis: A complete blood count is usually unremarkable except for a low white blood cell count and/or thrombocytopenia in up to one third of patients in the acute phase. Blood cultures on standard medium are invariably negative, as *Coxiella* will only grow in living cells or organisms. Most laboratories do not attempt cell culture as it poses a significant infection risk to laboratory personnel (BSL-3 precautions) and is often less sensitive than serology. Sputum examination is unremarkable even in patients with productive cough.

The bacterium utilizes few sugars, including xylose and glucose, so routine biochemical testing offers little informative information.

Commercial Identification systems.

None identified.

#### Immunoassay Tests.

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

**Two antigenic phases of *C. burnetii* infections exist: phase I (virulent) and phase II (avirulent).** Antibodies to phase II antigen is used to diagnose **acute** disease and antibodies to both phase I and phase II antigens to diagnose **chronic** disease. Antibodies to phase I and II antigens may persist for months or years after initial infection (12). Acute Q fever cases usually exhibit a much higher antibody level to phase II (first detected during the second week of illness). Antibodies to phase I antigens of *C. burnetii* generally take longer to appear and indicate continued exposure to the bacteria. In acute Q fever, patients will have IgG antibodies to phase II and IgM antibodies to phases I and II. Increased IgG and IgA antibodies to phase I are often indicative of Q fever endocarditis (13). Chronic Q fever is seen with antibodies to phase I antigens, generally requiring longer to appear and indicate continued exposure to the bacteria. High levels of antibody to phase I, in conjunction with constant or falling levels of antibody to phase II, suggest chronic Q fever.

Phase I variants of *C. burnetii* have a complete LPS structure and are virulent. In contrast, phase 2 variants have a truncated form of LPS, usually due to loss of one or more of the three sugars that comprise the LPS O-antigen (virenose, dihydrohydroxystreptose, and galactosaminuronyl- $\alpha$ (1, 6)-glucosamine). These variants grow very well in cell culture within host cells, but have impaired growth and reduced pathology when inoculated into animals (2, 1, 3, 4, 25, 37, 48).

Direct agent immunoassays.

Enzyme-linked immunosorbent assay (ELISA): The use of ELISA for agent identification has been reported to have a sensitivity down to 2500 organisms, an unfortunately high level of detection given the infectious dose of 1 to 10 organisms (59).

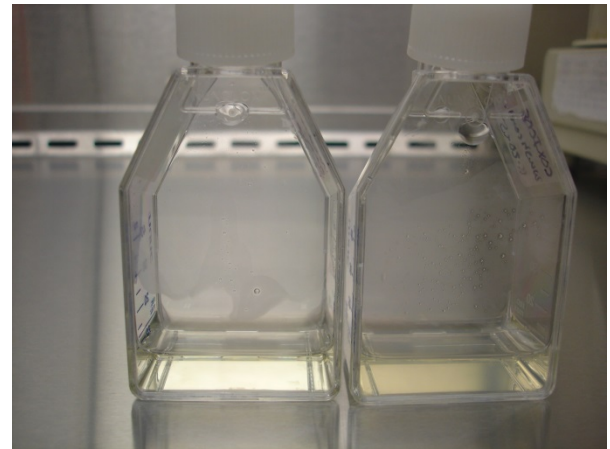


Figure 49. *C. burnetii* Nine Mile Phase I (COXI008) in ACCM-2 broth at day 19. Uninnoculated media control is on the left.

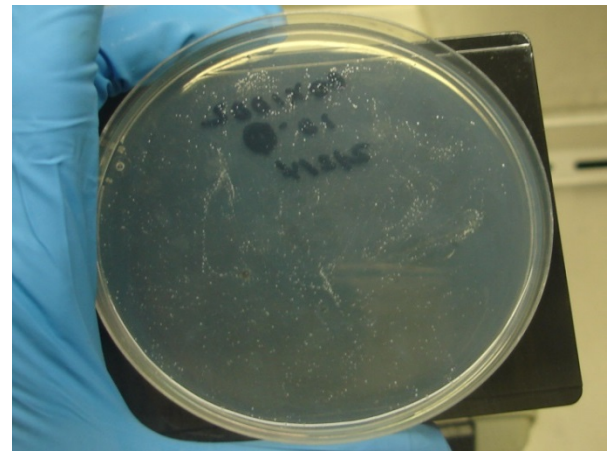


Figure 50. *C. burnetii* 9MII (COXI002) colonies on ACCM-2 agarose medium at 14 days.



Serological testing. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays. Serology is **most commonly** used to diagnose Q fever. Serological tests are confirmatory and include identification of antibody to *C. burnetii* by indirect fluorescent antibody, complement fixation (CF) (commonly used but somewhat insensitive), and ELISA (sensitive and easy to perform). Specific IgM antibodies may be detectable as early as the second week after onset of illness. Combined detection of IgM, IgA, and IgG antibodies improves assay specificity and provides accuracy in diagnosis. Cross reactivity with other organisms have been noted in the various serology tests (33, 38).

Indirect immunofluorescence (IFA): IFA, adapted as a micro-immunofluorescence technique, is another method for the serodiagnosis of Q fever. Serum dilutions are placed on immunofluorescence slides with wells coated with one or two antigens. If specific antibodies are present, they attach to the antigen on the slide and, following the addition of fluorescently conjugated anti-species-specific immunoglobulins, are then detected by examination with a fluorescence microscope (12).

Complement fixation (CF): CF antibody titer against phase I antigen equal to or greater than 1:200, or phase I IgA equal to or greater than phase II titer, is considered positive. The CF test may not be useful if sera have intrinsic anti-complement activity. A fourfold rise in CF antibody titer against phase II antigen is considered positive for acute disease but requires a baseline and repeat sample in 2-4 weeks (30).

Enzyme-linked immunosorbent assay (ELISA): The serological use of ELISA has high sensitivity and a good specificity. Ready-to-use kits are commercially available and can detect anti-phase II antibodies or both anti-phase I and II antibodies. ELISA is available at USAMRIID in which a single serum specimen can be used to reliably diagnose acute Q fever as early as 1 1/2 - 2 weeks into illness (24, 30).

Immunohistochemical (IHC) staining: *C. burnetii* have been identified in infected tissues by using immunohistochemical staining of paraffin-embedded tissues (45).

Direct fluorescent antibody (DFA) assays: Direct immunofluorescence assay with polyclonal anti-*C. burnetii* antibodies and an appropriate secondary antibody conjugated to fluorescein isothiocyanate (FITC) has been used. Specific monoclonal antibodies to phase I and phase II lipopolysaccharide are also available (29).

## Nucleic Acid Detection Tests.

General: The genome of *C. burnetii* contains a single 1,995,281 base-pair circular chromosome and four plasmids; QpH1, QpRS, QpDV, and QpDG, along with 29 chromosomal insertion sequences (51).

Polymerase chain reaction (PCR): Real-time PCR provides good detection of *C. burnetii* and is very useful in situations where culture is not practical (7, 9, 14, 22, 28, 32).

- Plasmids are 36-42 kb but share a 30 kb core (34).
- IS1111 is a multicopy insertion sequence, the most largely employed target. Inherent multi-copy numbers of this sequence allows the sensitivity of the test. The number of the insertion sequences (IS1111) varies widely (between 7 and 110) depending on the isolate (11, 32).
- Superoxide dismutase (sodB) gene (18, 56).
- com1 encoding a 27 kD outer membrane protein (19).
- Heat-shock operon encoding two heat shock proteins (*htpA* and *htpB*)
- Isocitrate dehydrogenase (*icd*).
- Macrophage infectivity potentiator protein (*cbmip*).
- Trans1 and trans2 transposon-like region.

A method for *C. burnetii* detection and differentiation between strains that cause endocarditis and those that cause acute Q fever, is based the difference between strains that contain unique plasmid sequences. Two sets of primers are required: the first derived from a fragment of plasmid QpH1 which has been detected in *all C. burnetii* isolates and a second using primers specific sequences shared only by QpRS plasmid-containing strains of *C. burnetii*. The first set detects the presence of *C. burnetii* and the second determines whether the isolate is associated with strains causing chronic disease (34).

Joint Biological Agent Identification and Diagnostic System (JBAIDS): The *Coxiella burnetii* (Q Fever) detection kit, when used with the JBAIDS platform (hardware and software), is capable of detecting *C. burnetii* organism. Specifically, this Q Fever test is based on a PCR assay that recognizes DNA target sequences present in *Coxiella burnetii*. For optimal assay performance, the DNA of the



pathogen has to be initially isolated from specimens using appropriate sample purification kits and procedures. This PCR-based test, however, does not distinguish between live or dead organism. This assay is FDA approved for clinical use in direct patient care.

### Other Diagnostic Methods.

None identified.

### Characterization

General: A number of discriminatory typing and characterization methods for molecular epidemiology of *C. burnetii* are in use, and often used together (8, 31).

16S rRNA sequencing: 16S-23S ribosomal DNA internal transcribed spacer (ITS) was used to try and characterize *C. burnetii*, but strains revealed very high levels of sequence similarity (> 99%) with different geographic origins and phenotypic characteristics. 16S-23S rDNA ITS sequencing is useful for identification but not applicable to characterization studies (57, 61).

PCR-restriction fragment length polymorphism (PCR-RFLP): PCR-RFLP was used to sort *C. burnetii* into six genomic groups (I to VI) based on 3 genes; *icd*, *com1* and *mucZ* (17, 20, 34, 55).

Beta-subunit of RNA polymerase (*rpoB*): PCR amplification of the *rpoB* gene and sequencing revealed fewer than four base differences and the distribution of the differences does not correlate with other genotypic groupings with the species (36).

Multi-locus variable number of tandem repeats analysis (MLVA): MLVA typing with 17 markers was described as having an increased resolution compared to IRS-PCR or MLST. MLVA analysis in one study differentiated 36 different genotypes. A 6 or 10 marker MLVA analyses was used in the 2007 human outbreak in the Netherlands to tie the outbreak to the source (dairy goat herds and 1 sheep herd). MLVA data can be queried on a dedicated MLVA genotyping web service (5, 42, 46, 54, 53, 58).

Randomly amplified polymorphic DNA analysis (RAPD): RAPD analysis of eleven isolates demonstrated that RAPD distinguished between MLVA related strains when a four primer set was used, but RAPD analysis is not commonly used yet (52).

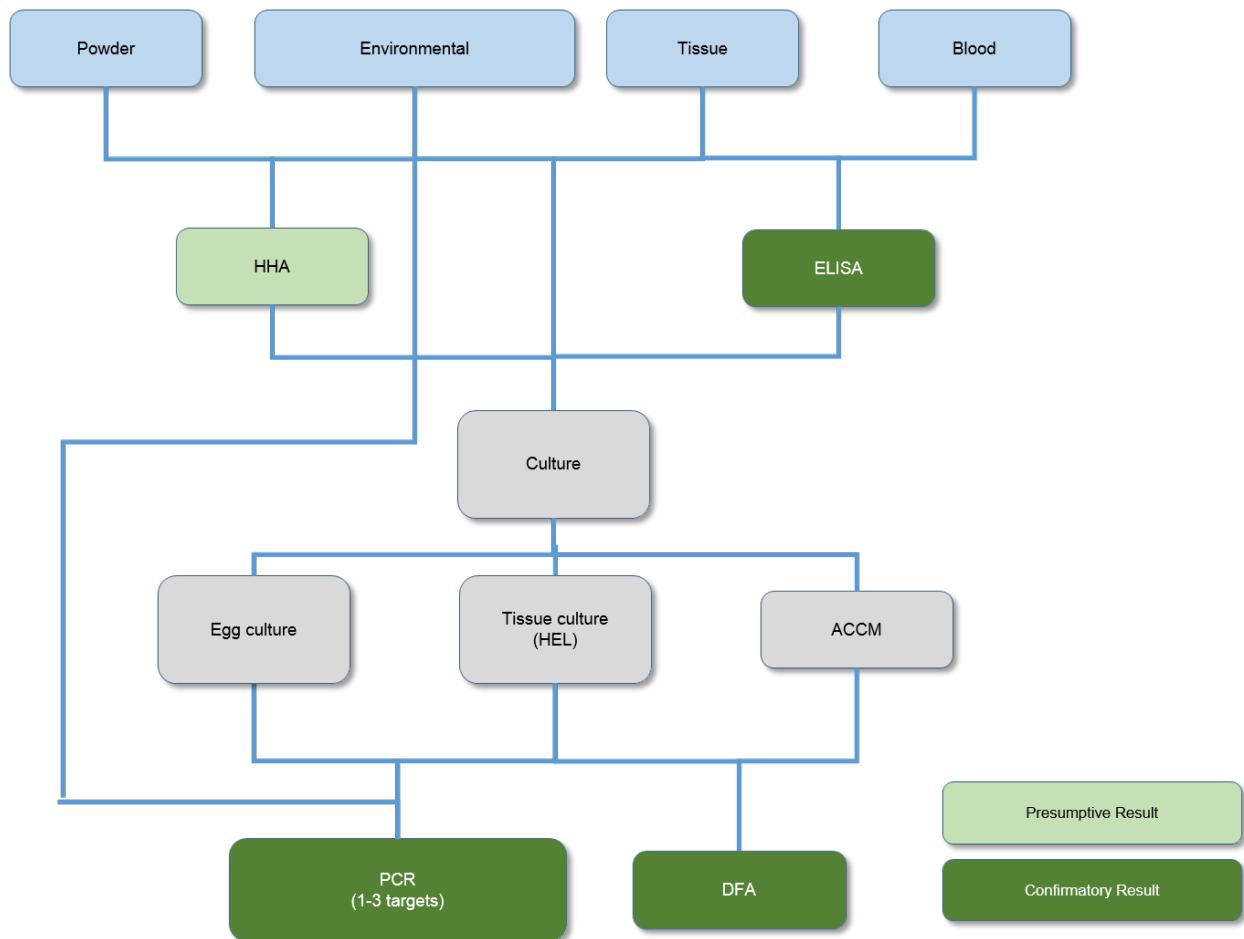
Infrequent restriction site-PCR (IRS-PCR): *C. burnetii* isolates were divided into six groups containing up to five different isolates by IRS-PCR in one study (5).

Pulsed-field gel electrophoresis (PFGE): Restriction enzymes *NotI* and *SfiI* gave the fewest and most easily resolved fragments. *SfiI* cuts the genome into 15 DNA fragments ranging in size from 320 to 18 kbp, and *NotI* cuts the DNA into 20 fragments from 293 to 10 kbp in size. 80 *C. burnetii* isolates derived from Europe, USA, Africa and Asia, restricted with restriction enzyme *NotI* yielded 20 different restriction patterns. The index of discrimination for this typing system was placed at 0.86. PFGE patterns revealed evolutionary relationships among groups that corresponded to the geographical origin of the isolates. This finding was confirmed by genetic mapping. But no correlation between restriction group and virulence of isolates has been detected (17, 23, 60).

Multispacer sequence typing (MST): Sequencing of ten genes (*Cox2*, *Cox5*, *Cox18*, *Cox20*, *Cox22*, *Cox37*, *Cox51*, *Cox56*, *Cox57*, *Cox61*) totaling approximately 5,000 base pairs when concatenated, contain highly conserved and hypervariable regions with single nucleotide polymorphisms (SNPs) as well as various insertions and deletions (indels). The hypervariable regions allowed for the discrimination and classification of the various isolates into different genotypes. There is a publicly available online database contains the sequences and MST groupings for approximately 170 isolates that were differentiated and represent 34-36 genotypes (16, 21, 53).

### Confirmation

Confirmatory diagnosis is by serology (indirect fluorescent antibody, complement fixation, ELISA), PCR, or *C. burnetii* positive cultures.



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# VIRAL AGENTS

## OVERVIEW

Viruses are the simplest of microorganisms and are intracellular parasites and lack a system for their own metabolism and reproduction. Viruses are much smaller than bacteria and vary in size from 0.02  $\mu\text{m}$  to 0.2  $\mu\text{m}$  (1  $\mu\text{m}$  = 1/1000 mm). Viruses consist of a nucleocapsid protein coat that can contain either RNA or DNA as their genetic material. In addition, the RNA viruses can be either single-stranded (ssRNA) or double-stranded (dsRNA). RNA viruses are further classified according to the sense or polarity of their RNA into negative-sense, positive-sense, or ambisense viruses. Positive-sense viral RNA is similar to mRNA and thus can be immediately translated by the host cell (see section on Alpha viruses below). As such, purified RNA of a positive-sense virus can directly cause infection and thus make many of these class of viruses select agents based on the nucleic acid alone (no intact cells). Negative-sense viral RNA is complementary to mRNA and thus must be converted to positive-sense RNA by an RNA polymerase before translation. Ambisense RNA viruses resemble negative-sense RNA viruses, except they also translate genes directly from the positive strand. In addition, viruses have many morphological shapes, helical (filamentous), icosahedral (spherical), or complex, including those with tails.

Viruses depend upon the metabolic systems of their host cells for metabolism and reproduction, meaning that they cannot be cultivated in synthetic media, but require living cells in order to replicate. Host cells that each virus will infect and replicate in are often different, even within the same family. Host cells can be from humans, animals, plants, or bacteria. Virus-specific host cells can be cultivated in synthetic nutrient solutions (tissue culture) or eggs, and then infected with the virus. After infection, virus replication can cause cells to lyse (lytic viruses) or not (temperate or lysogenic). Those viruses that do not cause cell lysis, may or may not cause visible cell changes in the host cells. Virus propagation is therefore expensive, time-consuming, and requiring more complex equipment and facilities. For this reason, virus culture is not typically considered a military fieldable capability.

## GENERAL INFORMATION

### Taxonomy Information.

Viruses are not easily placed on an evolutionary based tree of life and alternative taxonomic systems are commonly used. For viruses, a representation based on host maintenance is commonly used as well as two schemes: the Baltimore classification system, which places viruses into one of seven groups, and the International Committee on Taxonomy of Viruses (ICTV) system, which generally follows the Linnaean classification system, where organisms are in a ranked hierarchy.

Viral taxonomy can be based on host or function. Arbovirus is a non-precise term used to refer to a group of viruses that are transmitted by arthropod vectors. The word arbovirus is an acronym (ARthropod-BORne virus). Tibovirus (TICK-BORne virus) is sometimes used to describe viruses transmitted by ticks, a superorder within the arthropods. Arboviruses were once organized into alphabetic groups (A, B, C, D ...) but the number of groups would eventually exceed the length of the alphabet and the organization into groups has fallen out of usage.

The Baltimore classification places viruses into one of seven groups depending on the state of their nucleic acid (DNA or RNA), strandedness (single-stranded or double-stranded) with groups are designated by Roman numerals (1).

- Group I: Double-stranded DNA viruses; dsDNA viruses (e.g. Adenoviruses, Herpesviruses, Poxviruses)
- Group II: Single-stranded DNA viruses: ssDNA viruses (+ strand or "sense") DNA (e.g. Parvoviruses)
- Group III: Double-stranded RNA viruses: dsRNA viruses (e.g. Reoviruses)
- Group IV: Single-stranded RNA viruses - positive-sense:: (+)ssRNA viruses (+ strand or sense) RNA (e.g. Picornaviruses, Togaviruses)
- Group V: Single-stranded RNA viruses- negative-sense:: (-)ssRNA viruses (- strand or antisense) RNA (e.g. Orthomyxoviruses, Rhabdoviruses)
- Group VI: Positive-sense single-stranded RNA viruses that replicate through a DNA intermediate: ssRNA-RT viruses (+ strand or sense) RNA with DNA intermediate in life-cycle (e.g. Retroviruses)



- Group VII: Double-stranded DNA viruses that replicate through a single-stranded RNA intermediate: dsDNA-RT viruses (e.g. Hepadnaviruses)

ICTV's virus classification system uses a slightly modified version of the standard biological classification system. The current Linnaean classification system groups organisms from largest to smallest are: kingdom, phylum, class, order, family, genus, and species. ICTV uses a modification of the Linnaean classification system and only recognises the taxa order, family, *subfamily*, genus, and species.

Biothreat viruses constitute a small population of the viral world.

Smallpox is a member of the *Poxviridae* family of viruses that are DNA viruses. Poxviruses are divided into subfamilies based on host range with smallpox the most notable virus in the family. The high infectivity rate, coupled with its high mortality rate, continue to make smallpox a significant biothreat virus.

Alphavirus are positive sense, single-stranded RNA viruses. There are thirty alphaviruses able to infect various hosts including humans, birds, and larger mammals such as horses. Transmission between species occurs mainly via invertebrates, especially mosquitoes. Alphaviruses are therefore often part of the group of viruses termed arboviruses or arthropod-borne viruses. Alphaviruses tend to be spherical (although slightly pleomorphic) in form and are enveloped viruses. They typically measure about 70 nm in diameter and have a 40 nm isometric nucleocapsid.

*Arenaviridae*: *Arenaviridae* include the etiologic agents of Argentine, Bolivian, and Venezuelan hemorrhagic fevers, and Lassa fever. Lassa virus causes Lassa fever in West Africa, where endemic transmission is related to infected rodents. Nosocomial transmission is frequently a problem for health-care workers and has been attributed to contact with body fluids, and aerosols generated by patients. Argentine hemorrhagic fever (AHF) is caused by Junin virus. Bolivian, Brazilian, and Venezuelan hemorrhagic fevers are caused by the related Machupo, Guanarito, and Sabia viruses, respectively. These viruses are transmitted from their rodent reservoirs to humans, who inhale dusts contaminated with rodent excreta.

*Bunyaviridae*: *Bunyaviridae* include the members of the Hantavirus genus, the Congo-Crimean hemorrhagic fever virus from the Nairovirus genus, and the Rift Valley fever virus from the Phlebovirus genus. Congo-Crimean hemorrhagic fever (CCHF) is a tick-borne disease that occurs in the Crimea and parts of Africa, Europe, and Asia. It may also be spread by contact with infected animals, and in health-care settings. Rift Valley fever (RVF) is a mosquito-borne disease that occurs in Africa. The hantaviruses are rodent-borne viruses with a wide geographic distribution. Hantaan and closely related viruses cause hemorrhagic fever with renal syndrome (HFRS), (also known as Korean hemorrhagic fever or epidemic hemorrhagic fever). In addition, newly described hantaviruses cause hantavirus pulmonary syndrome (HPS) in the Americas. The hantaviruses are transmitted to humans via inhalation of dusts contaminated with rodent excreta.

*Filoviridae*: *Filoviridae* include Ebola and Marburg viruses. Ebola hemorrhagic fever was first recognized in the western equatorial province of the Sudan and the nearby region of Zaire in 1976. A related virus (Ebola Reston) was isolated from monkeys imported into the United States from the Philippine Islands in 1989. The African strains cause severe disease and death. While subclinical infections occurred among exposed animal handlers, Ebola Reston has not been identified as a human pathogen. Marburg epidemics have occurred on six occasions: five times in Africa, and once in Europe. The first recognized outbreak occurred in Marburg, Germany, and Yugoslavia, among people exposed to African green monkeys, and resulted in 31 cases with seven deaths. Filoviruses may be spread from human to human by direct contact with infected blood, secretions, organs, or semen. The natural reservoirs of the filoviruses are unknown.

*Flaviviridae*: *Flaviviridae* include dengue and yellow fever viruses. Yellow fever and dengue are two mosquito-borne fevers that have great importance in the history of military campaigns and military medicine. Tick-borne flaviruses include the agents of Kyasanur Forest disease in India, and Omsk hemorrhagic fever in Siberia.

<i>General viruses of biological warfare potential</i>					
<i>Alphaviruses Togaviridae</i>	<i>Arenaviridae</i>	<i>Bunyaviridae</i>	<i>Filoviridae</i>	<i>Flaviviridae</i>	<i>Poxviridae</i>
Barmah Forest virus complex	Lassa virus (Old World) complex Lassa fever virus Lujo virus	Hantavirus Hantavirus	Ebola Bundibugyo Reston Sudan Taï Forest Zaire	Flavivirus Dengue fever West Nile Yellow fever Japanese encephalitis Kyasanur Forest Murray Valley St. Louis encephalitis Tick-borne encephalitis	Orthopox smallpox virus (variola) vaccinia virus cowpox virus monkeypox virus
Eastern Equine Encephalitis complex	Tacaribe virus (New World) complex Junin Machupo Guanarito virus Sabiá virus	Nairovirus Crimean-Congo hemorrhagic fever virus (CCHFV)	Marburg Marburg virus Ravn virus		Parapox orf virus pseudocowpox bovine papular stomatitis virus
Venezuelan Equine Encephalitis complex		Orthobunyavirus La Crosse virus		Hepacivirus Hepatitis C Virus	Yatapox tanapox virus yaba monkey tumor virus
Western Equine Encephalitis complex		Phlebovirus Rift Valley fever		Pestivirus (non-human mammals)	Molluscipox molluscum contagiosum virus (MCV)
Middelburg virus complex		Tospovirus (plant viruses)			
Semliki Forest virus complex					
Ndumu virus complex					

## LABORATORY DIAGNOSTICS

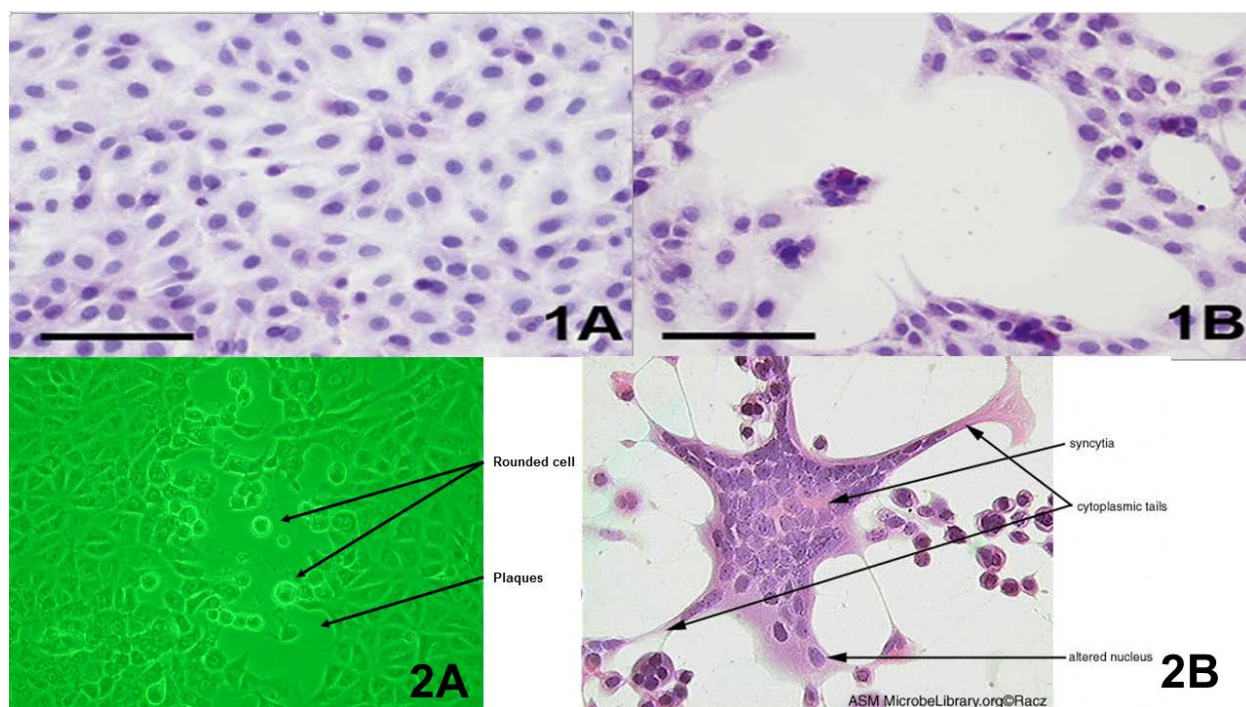
### General Diagnostic Information.

#### General.

Like the bacterial biothreat agents, being able to propagate the causative agent unequivocally demonstrates the pathogen's presence, viral culture serves the same purpose. However, viral culture is complicated and requires highly refined conditions to successfully propagate viruses. Virus culture is not typically contemplated in a deployed laboratory as the logistic and technical burdens are extensive.

#### Culture.

Cell culture is typically used most often due to the difficulty with egg manipulation and the inherent problems with handling infected animals. In using cell cultures, tissue fragments are first dissociated, usually with trypsin or collagenase, the cells placed in petri dishes, flasks, bottles, or test tubes with a liquid medium. Over time the cells will attach and spread on the bottom of the container and then start dividing, giving rise to a primary cell culture. Commonly employed cell lines include primary monkey kidney, vero, Hep-2, or human diploid (HEK and HEL). A virus typically brings about changes in the host cell that eventually lead to cell death or other distinguishing cytopathic effects. Some biowarfare or extremely hazardous viruses do not produce cell death cytopathic effects and require other methods to determine their presence in cell culture.



**Figure 51. Cytopathic effects; 1A- monolayer of cells; 1B- plaques formed by viral activity; 2A – cell cytopathic effect of cell rounding; 2B – cell cytopathic effects of syncytia, nucleation, and cell morphology changes.**

General viral information					
	Virus	Endemic area	Mortality	Cells and incubation time	Growth characteristics
Poxviridae	Variola	Prior to eradication in 1980 with vaccinia virus as vaccine it was endemic in Europe, North America, South America, and many part of Africa	30% ( range, 15-50%)	Vero E6- 4 days	CPE is observed; plaques fine
	Monkeypox	Central and West Africa	1-10%	Vero E 6 or BSC-1- 3 to 4 days	CPE is observed; plaques fine
	Orf virus	Europe and New Zealand	Benign; self-limiting	Primary ovine or bovine cells- 2 to 3 days	CPE is observed; plaques fine
	Tanapox	Kenya and Zaire	Benign; self-limiting	Owl Monkey Kidney cells and primary human dermal fibroblasts – 3 to 4 days	CPE is observed; plaques fine

	Molluscipox	Infections occur worldwide, but are more common in warm, humid climates where living conditions are crowded	Benign; self-limiting	Virus cannot be routinely cultured	PCR will confirm virus
Alphaviruses Togaviridae	Barmah Forest virus complex	Australia	Benign; self-limiting	BHK-21, C6/36 and Vero cells- 3 to 5 days	CPE is observed; plaques fine in Vero
	Eastern Equine Encephalitis complex	North, Central and South America, Caribbean	33%	Vero, A549 and MRC-5- 2 to 8 days	CPE observed in Vero by day 2 and in other cells by day 8; plaques fine in Vero
	Venezuelan Equine Encephalitis complex	North and South America	0.5 to 1%	Vero and BHK-21- 24 hours	CPE is observed; plaques fine in Vero
	Western Equine Encephalitis complex	North and South America	4%	Vero, DEF and C6/36	Plaques fine in Vero
	Middelburg virus complex	Africa	Benign; self-limiting	BHK-21, C6/36 and Vero cells- 18 hours	CPE is observed; plaques fine in Vero and BHK
	Semliki Forest virus complex	Africa	Benign; self-limiting	BHK-21, C6/36, CHO- 2 to 3 days	CPE is observed; plaques fine in BHK cells
	Ndumu virus complex	Africa	While antibodies have been found in humans from several African countries, no human illnesses have ever been associated with Ndumu	Vero cells-1-3 days	CPE is observed; plaques fine in Vero
Arenaviruses	Lassa virus	West Africa	1% to 2%	Vero E6 - Vero 3-5 days	No CPE - requires 2nd assay - plaques.
	Junin	Argentinean pampas	30%	Vero - 3-5 days	No CPE - requires 2nd assay, plaques are difficult but possible.
	Machupo	Bolivia	25% to 35%	Vero E6 - 3 to 5 days	No CPE Plaques fine.
Bunyaviruses	Crimean-Congo hemorrhagic fever virus (CCHFV)	Africa, SE Europe, Central	30% <0.5%	SM 3 to 14 days Possible to passage in E6, SW13 or CER cells after initial isolation but may require >1 blind passages.	Plaque assays just as difficult.
	Rift Valley fever	Asia, India Africa		Vero - 2 to 4 days	CPE Easy to plaque.

	Hanta-virus (Hantaan, Dobrava, Seoul, Puumala, Sin Nombre, Andes)	Europe, Asia, South America (rare)	5% for HFRS	Vero E6 - 10 to 14 days	No CPE - requires 2nd assay such as IFA or PCR. Often requires blind serial passages to isolate. Hard to plaque.
Filoviruses	Ebola virus	Africa, Philippines (Ebola Reston)	50% to 90% for Sudan/ Zaire	Vero E6 - 6 to 12 days	CPE Plaques fine.
	Marburg virus	Africa	23% to 70%	Vero E6 - 6 to 12 days	CPE Plaques fine.
Flavivirus	Yellow fever virus	Africa, South America	Overall 3% to 12%, 20% to 50% if severe second phase develops	MK2 cells (also BHK21) - 3 to 6 days	Little to no CPE - requires 2nd assay such as PCR or IFA to confirm. Plaques fine in Vero cells!
	Kyasanur Forest disease virus	Southern India	3% to 5%	Vero/Vero E6 SM - 3 to 6 days	CPE Plaques fine.
	Omsk Hemorrhagic Fever virus	Siberia	0.2% to 3%	Vero/Vero E6 SM - 3 to 6 days	CPE Plaques fine.

### Eggs.

Fertile hen eggs are a less expensive, less regulated substitute for laboratory animals, but again, may have a restricted virus use range that doesn't cover all the viruses being isolated and identified. While eggs are less logistically challenging than laboratory animals, special incubators and an egg light are needed.

### Immunoassay Tests.

Immunoassays for viruses have been extensively developed to augment viral propagation, serve as direct mechanism to detect viruses, and aid in serodignosis for detecting prior exposure. Assays such as the complement fixation test, hemagglutination inhibition test, ELISA, and immunofluorescence have all been developed under specific viruses. For a general discussion of the technics, see Appendix C (Diagnostic Technologies).

### Nucleic Acid Detection Tests.

Like immunoassays, nucleic acid testing for viruses has also been extensively developed to augment viral propagation as well as serve as direct detection of viruses. Methods, such as non-amplified nucleic acid probes (liquid-phase, solid-phase, and in situ hybridization [ISH]) (5), amplified nucleic acid techniques (consisting of polymerase chain reaction [PCR] and derivatives), loop-mediated isothermal amplification of DNA [LAMP] (3), and microarrays (2), exist in various applications and for different viruses.

A couple of things should be noted, however, when the discussion of nucleic acid based diagnosis is started. First and foremost, most of the assays developed are typically developed for research studies and often don't get 'validated' to an extent acceptable to the diagnostic community. Very few of the assays presented in this manual have had Food and Drug Administration clearance sufficient that they can be used to test patient samples in support of patient diagnostics. While physicians are free to practice the art of medicine and often use research methods in patient treatment, legally that is not supported. Second, interlaboratory accuracy/comparison clearly shows that nucleic acid assays are not a panacea for routine diagnostic work. Interlaboratory comparison of nucleic acid assays in Europe show a proficiency ranging from decent (87%) to not very good (27%), across 8 different PCR proficiency tests (4).

### Other Diagnostic Methods.

Electron Microscopy (EM): EM is a primary method used in virology. EM detection and identification of viruses is based on their characteristic cellular morphology. Like culture, visualization of the virus is better than indirect detection methodology. While visualization and speed are advantages, EM's limitation in addressing multiple specimens simultaneously, low sensitivity (around 100 virus particles per ml) for detection, non-distinct morphological appearances, and the expense of providing EM service (to include the required highly skilled personnel) are disadvantages. There are two types of EM



methods, direct or immunoelectron microscopy (IEM). With direct methods, thin sectioning and negative staining are used to visualize viral particles. IEM uses antibodies labelled with heavy metal particles (e.g. gold) that can be directly visualized. IEM is a means of increasing the sensitivity and specificity of EM and is particularly useful where the number of virus particles present is small or the antibodies target distinct moieties.

Animal inoculation. Animals Inoculation, in an attempt to amplify and indentify the viral agent, is commonly practiced, but involves more complexities than viral culture. Not only do you have to maintain animals, and address the corresponding logistics, but also since about 1971 whe the Animal Welfare Act was passed as law, animal use has been regulated and includes animal use reviews by an Institutional Animal Care and Use Committee. In addition, no one animal species is appropriate for all animal work with viruses. Mice have become the predominate animal model, but suckling mouse is more appropriate for some viruses (ie; arboviruses), and specific animals are sometimes used for more specific virus isolation (ie; avian influenza).

## Characterization

Unlike bacteria where characterization is based on phenotypic traits, virus characterization is predominately genomic based. While host range, electron microscopy morphologies, and serological/antigenic groupings are sometimes used, genomic variations are the principle characterization method. Viruses, with their smaller overall genome size are subject to genetic drift that will have a bigger impact on characterization.

## Confirmation

Currently, the only confirmation scheme published by the CDC LRN involves smallpox. Confirmation of the other viruses will be based on available technologies.

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# Poxviridae family

## OVERVIEW

The *Poxviridae* family consists of double-stranded, enveloped, DNA viruses that are capable of infecting both vertebrates and invertebrates. Poxviruses are divided into subfamilies based on host range. The name of the family, Poxviridae, is a legacy of the original grouping of viruses associated with diseases that produced poxes in the skin, however, if infection is via the respiratory route, a systemic infection will occur.

The smallpox virus (*Variola*) remains as the most notable member of the family due to its high infectivity rate and its potential for high mass casualties in biological warfare incident. Smallpox is caused by the DNA-containing *Orthopox* virus, *Variola*, which is known to exist in at least two strains, *Variola* major and the milder form, *Variola* minor. It is believed that the disease emerged in human populations about 10,000 BC, with the earliest physical evidence in a pustular rash on the mummified body of Pharaoh Ramses V of Egypt. Of all those infected, 20-60% can die from the disease. Smallpox was responsible for an estimated 300-500 million deaths during the 20th century. After the vaccination campaigns throughout the 19th and 20th centuries, the WHO certified the eradication of smallpox in 1980. There is a long history of smallpox being used in biological warfare. Aerosol release of variola virus by terrorist organizations is a severe threat.

## ORGANISM INFORMATION

### Taxonomy.

Within the family *Poxviridae*, there are two subfamilies; *Chordopoxvirinae* and *Entomopoxvirinae*. Four genera of the subfamily *Chordopoxvirinae*, *Orthopoxvirus*, *Parapoxvirus*, *Yatapoxvirus*, and *Molluscipoxvirus*, contain species that can cause lesions on human skin or mucous membranes with mild to severe systemic rash illness. Different references will have some different taxonomy representations.

#### FAMILY: Poxviridae

##### SUBFAMILY: *Entomopoxvirinae* (infect arthropods)

##### GENERA:

- Alphaentomopoxvirus
- Betaentomopoxvirus
- Gammaentomopoxvirus

##### SUBFAMILY: *Chordopoxviridnae* (infect vertebrates)

##### GENERA:

##### Avipoxvirus (fowlpox)

Avipoxvirus genus infects principally birds but the virus can infect humans who have close contact with birds that are infected. The virus can be transmitted through vectors such as mosquitoes.

##### Capripoxvirus (sheep-pox).

Capripoxviruses are among some of the most serious poxviruses. Sheep, goat, and cattle serve as natural hosts and humans are not usually affected. These viruses damage hides and wool, forcing trade restrictions resulting in economic losses. The genus consists of three species: the type species: sheeppox virus, goatpox virus, and lumpy skin disease virus.

##### Leporipoxvirus (myxoma).

Lagomorphs and squirrels serve as natural hosts. There are currently four species in this genus including the type species *Myxoma* virus.

##### Molluscipoxvirus (molluscum contagiosum virus (MCV)).

The virus affects only the outer (epithelial) layer of skin and in healthy people it does not circulate throughout the body. Molluscum contagiosum causes raised, pearl-like papules or nodules on the skin. Molluscum infections occur worldwide but are more common in warm, humid climates and where living conditions are crowded. There are 4 types of MCV, MCV-1 to -4. MCV-1 is the most prevalent in human infections.

##### Parapoxvirus (milker's nodule).



Parapoxviruses consist of orf, pseudocowpox, and bovine papular stomatitis virus. Orf infects primarily fingers, hands, arms and the face. It has been recorded since the late 19th century from most sheep or goat-raising areas in Europe, the Middle East, the United States, Africa, Asia, South America, Canada, New Zealand and Australia. This disease has an economic impact on farmers worldwide. The mortality rate related to orf is usually low, but it may be very high in small ruminants, especially when bacterial or fungal secondary infections occur. Pseudocowpox is a common, mild infection of the udder and teats of primarily cows, but also sheep and goats, and is widespread worldwide. Reddish, raised, sometimes ulcerative lesions can also occur on the lips, muzzle, and in the mouth of the animal. The virus can also cause lesions on the hands of milkers. Bovine papular stomatitis virus infection is associated with nodules and pustules on the hands and sometimes on the face of milkers. Diseases with parapoxviruses are economically important leading to a decrease in milk production and interruption of lactation in many cows.

Yatapoxvirus (tanapox and Yaba monkey tumor virus).

Human tanapox was first seen among individuals in Kenya along the flood plain of the Tana River in 1957 and 1962. It is an acute febrile illness accompanied by localized skin lesions. While it has been mostly documented in Kenya and Zaire, it is believed to occur much more widely throughout tropical Africa. Yaba monkey tumor virus was first diagnosed in a colony of captive Asian rhesus monkeys and a baboon in Yaba, Nigeria. This virus produces a very distinct disease in primates that is characterized by epidermal histiocytomas of the head and limbs.

#### **ORTHOPOXVIRUS** (Variola, Vaccinia, cowpox, monkeypox)

##### Variola.

From a biothreat perspective, smallpox must be distinguished from other similar viral infections, such as vesicular exanthems, such as varicella zoster virus (VZV; chickenpox), herpes simplex virus (HSV), non-infectious erythema multiforme with bullae, or allergic contact dermatitis. After the first confirmation of smallpox, testing would likely be based on a clinical diagnosis rather than requiring laboratory confirmation of each case. In people with partial immunity due to previous vaccination, relatively mild cases of smallpox would be problem in recognizing the correct signs and symptoms.

##### Vaccinia Virus

Vaccinia virus is the current smallpox vaccine virus; it does not exist in nature and its origins are not known, but historic virus collections do suggest that it was originally a naturally occurring virus or mixture of predecessors (18). Although vaccinia is used as the smallpox vaccine, and had been used for expression of proteins for a variety of other viruses, complications from vaccination are a problem (5, 25). Generalized, progressive, eczema, and postvaccinal encephalitis have been described (3). Patients with vaccinia virus infections present with skin manifestations and usually have live viral particles replicating in the dermal lesions. The presence of vaccinia virus can be confirmed by obtaining a biopsy of the skin lesion, with examination through microscopy, plaque titer assay, Western blot, or nucleic acid amplification. The diagnosis of vaccinia virus complications is usually straightforward and depends on obtaining the history of recent vaccinia virus exposure by vaccination or contact with a vaccinated individual. Vaccinia necrosum (gangrenosa), also known as progressive vaccinia, is the most severe complication of vaccinia inoculation. Vaccinia necrosum is due to the accidental or inadvertent administration of vaccinia virus to immunocompromised individuals. Eczema vaccinatum occurs in patients with a history of eczema, who are unusually susceptible to infection with both the herpes simplex virus and vaccinia virus. The virus multiplies rapidly in eczematous skin. Lesions begin to appear at distant sites as the virus spreads throughout the body. Postinfection encephalitis is a rare and serious complication of infection with several viruses, including measles and vaccinia. The diagnosis of CNS complications is more difficult because the signs and symptoms are nonspecific. Although rare, postvaccinal encephalitis should be considered in any patient with neurologic symptoms developing 1-2 weeks after exposure to live vaccinia virus (37).

Cowpox Virus

Cowpox virus has a broad range of animals it can infect, including cows and humans. Cowpox virus has been described as the closest virus to the ancestral poxvirus from which the other orthopox viruses have evolved (14).

Monkeypox Virus

Monkeypox virus is one of the more divergent of the orthopox viruses (14). It affects humans with a 11-15% mortality rate (18, 43). The 2003 outbreak in the US infected 81 people. It was traced to the import of Gambian rodents that infected prairie dogs that were being sold as household pets (22, 24). The sequence of the prairie dog strain is distinct from the virus in central Africa that causes mortality there (44).

**Epidemiology and Endemic Area**

Species of the Genus Orthopoxvirus			
Species	Animals Infected	Host Range	Geographic Range
Variola	Human	Narrow	Formerly worldwide
Vaccinia	Human, <sup>a</sup> cow, pig, buffalo, rabbit, etc.	Broad	Worldwide <sup>b</sup>
Cowpox	Rodent, a cow, human, cat, etc.	Broad	Europe
Monkeypox	Squirrel, <sup>a</sup> monkey, ape, human	Broad	Western and central Africa
Ectromelia	Mouse, mole	Narrow	Europe
Camelpox	Camel	Narrow	Africa and Asia
Taterapox	Gerbil	Narrow	Western Africa
Volepox	Vole	?	United States
Raccoonpox	Raccoon	?	United States
Skunkpox	Skunk	?	United States
Uasin Gishu	Horse	Medium	Eastern Africa

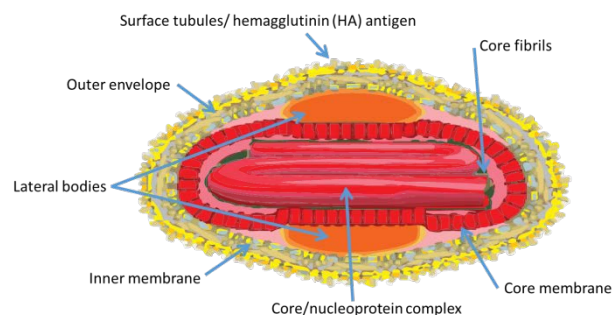
<sup>a</sup>Primary host. / <sup>b</sup>Secondary to vaccination; no known natural host.

**LABORATORY DIAGNOSTICS****Biosafety Information.**

"Worldwide, all live variola virus work is to be done **only** within WHO approved BSL-4/ABSL-4 facilities; one is at the CDC in Atlanta and the other is at the State Research Center of Virology and Biotechnology (VECTOR) in Koltsovo, Russia."

**Diagnostic Information.****General.**

Orthopox viruses are an enveloped, brick-shaped virus, 250nm long and 200nm wide, with a linear dsDNA genome of 170-250kb. The linear genome is flanked by inverted terminal repeat (ITR) sequences, which form covalently closed hairpin termini each end. Surface tubules or surface filaments are present on the surface membrane. Orthopoxvirus produce hemagglutinin (HA) antigen. The HA is a good immunoassay and nucleic acid based



**Figure 52. Cartoon of Orthopoxvirus structure.**  
Orthopoxvirus produce hemagglutinin antigen that serve as diagnostic markers.

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assay target. While the HA is specific for each strain, immunoassays are rarely able to be developed for strain differentiation.

Pox viruses are unique enough that specimens and specimen collection are often key to the diagnosis of the virus. Scabs, vesicles or pustules are primary samples for submission to the laboratory. Electron microscopy of vesicle fluid or scab extracts is the most rapid and useful technique to aid in diagnosis. Specimens can be collected using forceps to pick off a scab, the blunt edge of a scalpel to open vesicles or pustules, or a cotton swab to absorb the fluid. Typical variola specimens might include scrapings of skin lesions, lesion fluid, crusts, blood, or pharyngeal swabs (17, 18).

Under a light microscope, aggregations of virus particles, called Guarnieri bodies, can sometimes be seen. These characteristic cytoplasmic inclusions are the sites of viral replication and are readily identified in skin biopsies stained with Gispén's modified silver stain or hematoxylin and eosin and appear as pink blobs.

From a biothreat perspective, smallpox must be distinguished from other similar viral infections, such as vesicular exanthems, such as varicella zoster virus (VZV; chickenpox), herpes simplex virus (HSV), non-infectious erythema multiforme with bullae, or allergic contact dermatitis. After the first confirmation of smallpox, testing would likely be based on a clinical diagnosis rather than requiring laboratory confirmation of each case. In people with partial immunity due to previous vaccination, relatively mild cases of smallpox would be problem in recognizing the correct signs and symptoms.

#### Culture.

Most orthopox viruses, except, molluscum contagiosum virus, can be grown in cell culture and assayed by plaque counts. Species with a restricted host range, such as variola virus, replicate in a narrower range of cell culture cell lines, but serial passage adapts the virus and may invoke a more lytic plaque. Vaccinia, cowpox, and monkeypox are less likely to produce plaques and usually yield much less virus than the more lytic pox viruses. Differential growth and plaque sizes in particular cell lines may be useful in distinguishing between viruses when these viruses are first inoculated into such cells; however, adaptation occurs readily.

Culture of orthopox viruses		
Species	Cell line for culture	Comments on culture
Variola (Smallpox)	Definitive diagnosis of variola has classically required isolation of the virus and characterization of its growth on chicken egg chorioallantoic membrane or in cell culture.  Grows readily on many cell lines such as vero, HeLa, human embryonic lung, rhesus monkey kidney, SF, LL-MK <sub>2</sub> , and MRC-5.	Culture of variola requires biosafety level-4 and, under a World Health Organization monitoring, and has been restricted to only two secure laboratories; SRC VB Vector (Variola Virus Maximum Containment Laboratories to the State Research Centre of Virology and Biotechnology, Koltsovo, Novosibirsk Oblast, Russian Federation) and CDC (Variola Virus Maximum Containment Laboratories Atlanta, Georgia) (27). Cell cytopathic changes include multinucleation, cell rounding with cytoplasmic extensions and small plaques. More recently, definitive laboratory identification of variola virus involves growing the virus in cell culture and the use of PCR (39). Nucleic acid amplification is now a method of choice for more rapid identification (18). Restriction fragment-length polymorphisms analysis has also been included to differentiate subspecies and virus identity as part of definitive identification (18, 40). Chicken egg chorioallantoic membrane pocks are ~1 mm at 72 hrs, grayish white, opaque, dome shaped and not hemorrhagic (32).
Vaccinia	Easily grown in most cell lines, including vero, HeLa, human embryonic lung, rhesus monkey kidney, SF, LL-MK <sub>2</sub> , and MRC-5.	Cells fuse and fall apart forming 2-6 mm plaques and cytoplasmic bridging. Chicken egg chorioallantoic membrane pocks are 3-4 mm at 72 hrs, flattened with central necrosis and ulceration (32).
Cowpox	Grows readily on many cell lines or in chick chorioallantoic membranes where characteristic hemorrhagic pocks are produced.	Chicken egg chorioallantoic membrane pocks are 2-4 mm at 72 hrs, flattened and round with a bright red center (red cells) (32).
Monkeypox	Grows readily on many cell lines	2-6 mm plaques but no cytopathic in embryonic pig

		kidney cells Chicken egg chorioallantoic membrane pocks are ~1 mm at 72 hrs, flat and ridged on periphery with a crater center, some hemorrhage usually present (32).
Ectromelia		
Camelpox		
Taterapox		
Volepox		
Raccoonpox		
Skunkpox		
Uasin Gishu		

#### Bovine Papular Stomatitis Virus

Madin–Darby bovine kidney and vero (African green monkey kidney epithelial) cells with blind passages of 5 days each have been used looking for cytopathic effects. While isolation from humans has not been done, the characteristics of the lesions and the association between infected animals and the human case can be use for a causal diagnosis (7).

#### Molluscum Contagiosum Virus

Diagnosis is made on the clinical appearance, as the virus cannot routinely be cultured (38).

#### Orf Virus

Primarily a disease of goats or sheep, human infections can result in a single lesion on fingers, hands, arms, or the face (51). For virus isolation, primary ovine or bovine cells are used (47).

#### Pseudocowpox Virus (milker's nodule or paravaccinia)

Pseudocowpox is normally a cattle disease but lesions on hands or face has been reported. The virus can be propagated in some cell cultures but cannot be grown in fertile eggs (13). Scabs examined with an electron microscope will show virus particles.

#### Tanapox Virus

Owl Monkey Kidney (OMK) cells and primary human dermal fibroblasts are used virus propagation (29, 33).

#### Yaba Monkey Tumor Virus

No known human natural infections have been reported but similar tumors have been seen in experimental transmission. Of the cell cultures tested, only cynomolgus monkey kidney cell line JINET cells were characterized by focal multi-layering of cells and lipid accumulation in the cytoplasm of infected cells (50).

### **Immunoassay Tests.**

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

#### **Direct agent immunoassays.**

Lateral flow immunochromatography assays (LFIA/LFT/LFA), hand-held assays (HHA), “Smart tickets”: Lateral flow assays are commercially available along with some governmental assays for smallpox/orthopox (49)

Enzyme-linked immunosorbent assay (ELISA): An orthopox genus-specific antigen capture ELISA was developed based on a monoclonal capture antibody and rabbit hyper-immune sera detected with anti-rabbit horseradish-peroxidase- (HRP) conjugate and 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. The detection limits varied in a wide range of  $10^2$ - $10^4$  TCID<sub>50</sub>/ml., depending the individual virus strains. (21)

Electrochemiluminescence (ECL): An ECL assay, based on the legacy M1M system, was

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developed for orthopox with a limit of detection (LOD) of  $1.25\text{--}6.2 \times 10^6$  pfu/ml but some crossreactivity was seen with strains of *S. aureus*, *B. thuringensis*, *B. mycoides*, kaolin, clay and loamy soil and a number of sera from dogs, donkey, guinea pig, mouse and rabbit (11% false-positive rate). The detector and capture antibodies were a combination of 10 different monoclonal antibodies for an orthopox assay; not species specific (5, 25, 26).

Serological testing. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays. Hemagglutination inhibition, radioimmunoassays, ELISA, indirect immunofluorescent, complement fixation and neutralizing tests in cell culture or egg culture have all been used.

Radioimmunoassays (RIA): RIA assays have fallen out of favor due to the radioactive tags used, but RIA assays for vaccinia or monkeypox serology were developed and shown to be better than complement fixation. Anti-rabbit  $^{125}\text{I}$ -labeled globulin was used in the study, but a human based assay has also been developed (3, 19, 52).

Hemagglutination test (HAI or IHAT): Hemagglutination inhibition assays have been used but do not differentiate the various pox viruses since the hemagglutination is common among the orthopox viruses (3).

Enzyme-linked immunosorbent assay (ELISA): Orthopox viruses share cross homologous proteins that make specific antigenic ELISA assays difficult to establish. Using various combinations of monoclonal antibodies and polyclonal antibodies has been done with fair sensitivities but caution should be used in the case of limited validation data. Genus specific ELISA, with no claim for specific virus detection, have been developed and are useful as part of a screening diagnostic assay (6, 21). ELISAs to parapox, tanapox, and monkeypox (8, 15) and orthopox (vaccinia virus) (3, 28, 31). A variola virus-specific immunoglobulin and antigen ELISAs has also been developed (27). Differentiation of specific antibodies to each virus probably is not possible due to antigenic overlaps. In one orthopox serological ELISA study, it was done in Brazil to evaluate the seroprevalence of orthopoxviruses in a rural area where no orthopoxvirus outbreaks have yet been reported. An overall seroprevalence of 27.89% was found, and it was 23.38% in a non-smallpox vaccinated population. The results strongly suggested that one of the orthopox viruses was circulating in that population (31).

Plaque reduction neutralization test (PRNT): PRNT is considered the gold standard for detecting and quantifying many virus antibody titers, including vaccinia virus (4). Analogous to PRNT is a neutralization tissue-culture enzyme immunoassay that can be used in place of PRNT for vaccinia virus-neutralizing antibodies in sera of vaccines (11, 42).

Indirect fluorescent antibody (IFA): IFAs to most of the pox viruses have been developed, but again, within genus specificity probably is lacking due to antigenic overlap. The test has been reported useful in the diagnosis of monkeypox, vaccinia, and cowpox after 2-4 weeks from disease onset (3).

Western blot assay: Western blot assays can be specific if the antigens are highly purified, especially by removing cell culture serum components that enhance cross-reactivity. While potentially a good technique for serological evaluations, few laboratories use this method since a reliable standardization is not available (18, 48).

### Nucleic Acid Detection Tests.

Several nucleic acid techniques have been developed for specific poxvirus identification, with nucleic acid amplification by polymerase chain reaction (PCR) becoming the most widely used method. A generic *Orthopoxvirus* assay uses a conserved target is being used as a screening test. Real-time PCR methods for vaccinia virus, based on the DNA polymerase E9L gene has been developed, validated, and deployed, but some of these assays are specific for just vaccinia virus and will detect other poxviruses. In North America, a positive test is considered diagnostic for vaccinia virus unless medical or epidemiologic evidence suggests otherwise. With slight modifications to the probe, this assay is also used to detect variola virus.

There have been a number of PCR assays developed for detection of smallpox and the other pox viruses (18):

Genus	Target	Reference
<b>Orthopoxvirus</b>	HA/A56R, F4L, F89, vgf	(2, 1, 20, 34, 36, 40, 41, 45)
<b>Variola</b>	HA/A56R, 7R3U, 7R3L, A27L, A38R,	(9, 30, 41, 45, 46)
<b>(Smallpox)</b>	B9R, B10R, B11R, B12R,	
<b>Vaccinia</b>	B10R,, C9L	(10, 23, 45)
<b>Cowpox</b>	D11L, B9R	(35, 42).
<b>Monkeypox</b>	B7R, E5R	(26, 45)
<b>Sheep-pox</b>	P32	(16)
<b>Parapox</b>	B2L	(12)

#### Other Diagnostic Methods.

Electron microscopy (EM): Using electron microscopy with negative staining, visualization of the characteristic, large brick shape, orthopoxviruses identification is easy, but discrimination of variola from vaccinia, monkeypox, or cowpox, is not possible. A presumptive diagnosis can be based EM of vesicular scrapings or other samples. If a non-pox virus is seen on EM, like herpesviruses, this will eliminate smallpox and other orthopoxvirus infections from the diagnosis (3, 18, 41).

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# Alphaviruses; Family *Togaviridae*

## OVERVIEW

The genus *Alphavirus* is a member of the *Togaviridae* family, a diverse group of principally mosquito-borne RNA viruses that cause a variety of diseases worldwide. Alphaviruses are therefore often part of the group of viruses termed arboviruses, or arthropod-borne viruses. The pathogenic alphaviruses can be divided into those that cause a rash and arthritis (mainly the Old World alphaviruses) and those that cause encephalitis (New World alphaviruses). Alphaviruses are responsible for several medically important emerging diseases, are significant veterinary pathogens, and many infect humans. As a genus, the alphaviruses are widely distributed through the world, inhabiting all of the continents except Antarctica. The geographic distributions of individual species are restricted because of specific ecological conditions and reservoir host and vector restrictions. Members of the genus *Alphavirus* are typically maintained in natural cycles involving transmission by an arthropod vectors among susceptible vertebrate hosts. Virus-host interactions may be highly specific, and sometimes only a single mosquito species is utilized as the principal vector of a virus, as has been reported for many of the Venezuelan equine encephalitis (VEE) complex viruses. In contrast to their relationships with mosquito vectors, individual alphavirus species may use several different vertebrates as enzootic hosts.

The aerosol infectivity and their ability to cause severe, sometimes fatal neurologic diseases, make some of the alphaviruses militarily important. Alphaviruses of greatest importance as potential biological weapons include eastern (EEEV), western (WEEV), and Venezuelan equine encephalitis (VEEV) viruses.

Venezuelan equine encephalitis (VEE) virus complex is a group of eight mosquito-borne viruses that are endemic in northern South America and Trinidad with occasional presence in Central America, Mexico, and Texas. VEE can cause severe disease in humans and equid (horses, mules, burros, and donkeys). In nature, a variety of mosquitoes transmits the disease, with equid animals serving as amplifying hosts and sources of the mosquito infection. While VEE viruses are not considered stable in the environment and heat and standard disinfectants can easily kill the virus, they are still considered a militarily effective biowarfare agent owing to the low infective dose; approximately 10-100 organisms (13, 20). While initially isolated in around 1933-1938, between 1969 and 1971, an epizootic of a "highly pathogenic strain" of VEE emerged in Guatemala, moved through Mexico, and entered Texas in June 1971. In 1995, the Venezuela and Columbia outbreaks involved over 75,000 human cases and as many as 300 deaths. VEE is better characterized than EEE or WEE, primarily because it was tested as a biowarfare agent during the U.S. offensive program. Other countries have also been, or are suspected to have, weaponized this agent. The VEE virus is relatively stable during the storage and manipulation procedures necessary for weaponization (16, 50).

Western and eastern equine encephalitis viruses are similar to the VEE complex, are often difficult to distinguish clinically, and share similar aspects of transmission and epidemiology. There is no evidence of direct human-to-human or horse-to-human transmission. Natural aerosol transmission is not known to occur.

## ORGANISM INFORMATION

### Taxonomy.

Alphaviruses and Rubiviruses are the two genera in the *Togaviridae* family of viruses with Alphaviruses the predominate biowarfare concern. The *Togaviridae* have a linear, non-segmented, single-stranded, positive sense RNA genome.

There are currently 28 Alphaviruses, grouped into 7 antigenic complexes based on their serological cross reactions (7, 54). These complexes are:

Barmah Forest - first isolated in Northern Victoria, Australia in 1974.

Eastern equine encephalitis - first isolated from infected horse brains in 1933.

Middleburg - first isolated in 1957 in the Eastern Cape Province in South Africa.

Ndumu - First isolated in 1959 in South Africa.

Semliki Forest - First isolated in Uganda in 1944.

Venezuelan equine encephalitis - First isolated in Venezuelan in 1938.

Western equine encephalitis - First isolated in 1930 in the San Joaquin Valley of California.

Often, the alphaviruses, bunyaviruses and flaviviruses get lumped into a group known as the arboviruses (viruses that are borne by arthropods; primarily mosquitos and ticks). While this works in some references, this manual will address them under the standard taxonomy method.

### Epidemiology and Endemic Area

Humans, mammals, birds, and mosquitoes serve as natural hosts. Transmission between species occurs mainly via invertebrates, especially mosquitoes.

Virus	Arthropod Vectors	Epidemiology and Endemic Area
<b>Barmah Forest Virus</b>	<i>Culex</i> and <i>Aedes</i> genera are primarily involved in transmission along the inland and coastal areas in Queensland, respectively.	Virus is currently found only in Australia where the disease is usually non-fatal; most infected people recover. Fever is self-limiting, but joint and muscle pain can persist for up to 6 months or longer.
<b>Semliki Forest Virus</b>	<i>Aedes aegypti</i>	Virus is found throughout Africa and parts of Asia. Most infections are very mild; however, there has been one reported death after laboratory infection.
<b>Middleburg Virus</b>	<i>Aedes caballus</i>	Human morbidity or mortality is not well determined.
<b>Ndumu Virus</b>	<i>Mansonia uniformis</i>	Ndumu has been found throughout Africa where antibody response to the virus has been identified. No human illnesses have been associated with Ndumu virus.
<b>Western Equine Encephalitis Virus (WEEV)</b>	<i>Culex</i> and <i>Culiseta</i> genera <i>Aedes albopictus</i> in Argentina	Overall mortality of WEEV is low (approximately 4%) and is associated mostly with infection in the elderly. Sporadic outbreaks have occurred in 1941 with more than 3,400 human cases, 277 human cases in 1975, 41 human cases in 1977, and 37 human cases in 1987. It is often found predominately in states west of the Mississippi River and is most common between April and September, with peaks in July and August. A subtype of WEEV in Argentina is likely endemic in South America (37).
<b>Eastern Equine Encephalitis Virus (EEEV)</b>	<i>Culiseta melanura</i> <i>Aedes vexans</i> are possible bridge vector in the northeastern US	EEEV infection can result in one of two types of illness, systemic or encephalitic. Approximately a third of those who develop EEE die. It is present in North, Central and South America and the Caribbean. In the US, an average of <10 human cases of EEE are reported annually. Most cases have been reported from Florida, Georgia, Massachusetts, New Jersey, and has extended north into New Hampshire and Maine
<b>Venezuelan Equine Encephalitis Virus (VEEV)</b>	<i>Culex</i> genera	Human infective dose is ~10-100. The virus is endemic in northern South America, and has spread throughout most of Central America, Latin America, the Caribbean, and Mexico. Some epidemics have affected ≥100,000 people with as many as 300 deaths (15).

## LABORATORY DIAGNOSTICS

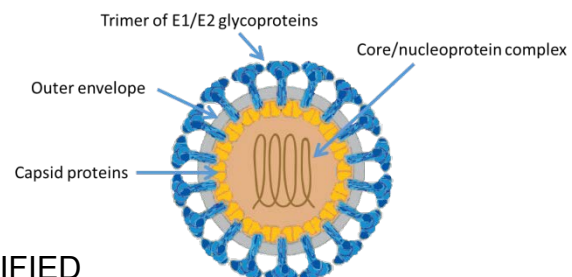
### Biosafety Information.

Various Alphaviruses have different biosafety recommendations.

NAME	RECOMMENDED BIOSAFETY LEVEL	BASIS OF RATING
Aura	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Babanki	2	Placed at this biosafety level based on close antigenic or genetic relationship to other viruses in a group of 3 or more viruses, all of which are classified at this level.
Barmah Forest	2	Placed at this biosafety level based on close antigenic or genetic relationship to other viruses in a group of 3 or more viruses, all of which are classified at this level.
Bebaru	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Cabassou	3	Insufficient experience with virus in laboratory facilities with low biocontainment
Chikungunya	3	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Eastern Equine Encephalitis	3 agricultural importance by the USDA	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Everglades	3	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Fort Morgan	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Getah	2	Disease in sheep, cattle or horses
Highlands J	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Kyzylagach	2	Insufficient experience with virus in laboratory facilities with low biocontainment
Mayaro	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Middelburg	2	Disease in sheep, cattle or horses
Mucambo	3	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Ndumu	2	Disease in sheep, cattle or horses
O'Nyong-Nyong	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Pixuna	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Ross River	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Sagiyama	2	Disease in sheep, cattle or horses
Semliki Forest	3	Fatal human laboratory infection – probably aerosol.
Sindbis	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Tonate	3	Insufficient experience with virus in laboratory facilities with low biocontainment
Trocara	2	Insufficient experience with virus in laboratory facilities with low biocontainment
Una	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Venezuelan Equine Encephalitis	3 agricultural importance by the USDA	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Western Equine Encephalitis	3	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
Whataroa	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations

### Diagnostic Information.

Alphaviruses are positive sense, single-stranded, enveloped, RNA viruses. Alphaviruses tend to be spherical (although slightly pleomorphic) in form and typically measure about 70 nm in diameter and have a 40 nm isometric nucleocapsid. The diagnosis



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of alphavirus infections generally relies on isolation of virus in the serum or cerebrospinal fluid during the acute phase of disease. Detection of IgM during the acute phase, or the seroconversion of individuals between acute and convalescent phases are also used for diagnostics. Sera at 2- to 3-day intervals is assessed upon early suspicion of a viral encephalopathy consistent with an alphavirus.

Current Centers for Disease Control and Prevention (CDC) guidelines (35) for diagnosis of an alphavirus (arbovirus) infection require (1) an acute febrile illness with encephalitis during a time when transmission of the virus is likely and (2) one more of the following criteria:

- A greater than 4-fold increase in the viral antibody titer between acute and convalescent sera (often 10 wk. apart)
- Viral isolation from the CSF, blood, or tissue
- Immunoglobulin M (IgM) positive to the organism in the CSF

The use of immunoassays (e.g., hemagglutinin inhibition, immunofluorescence, neutralization, complement fixation) are valuable for diagnosis. Presumptive positive diagnoses can be made based on the immunoassays or nucleic acid amplification assays.

#### Culture.

Alphaviruses can be propagated in vero cells, or other cells based on the virus, but optimal recovery of virus from a patient differs based on the stage of illness.

#### Barmah Forest Virus

This virus is only found in Australia and thus, a key in diagnosing this viral infection. The virus can be propagated on BHK-21 or C6/36 cell culture. Cytopathic effect was evident approximately 36 hours post-inoculation in the BHK-21 cells but took upwards of 5 days in the C6/36 cell line (26).

#### Chikungunya

Chikungunya virus (CHIKV) is a mosquito borne alphavirus that is causing large scale epidemics in a number of countries. Recent increased transmission has been driven by the emergence of an African lineage with enhanced transmission and dissemination in *Aedes* mosquito hosts. Two main genotypes of the CHIKV virus are characterized by the presence of a substitution of a valine for an alanine at position 226 of the E1 protein. An increasing number of cases are a non-classical presentation that includes encephalitis and meningitis. In HeLa cells the recent valine isolate showed less infectivity and the *Aedes albopictus* C6/36 cell line was significantly more permissive for virus growth (55).

#### Eastern Equine Encephalitis

RD (human embryonal rhabdomyosarcoma) and vero cells both show cytopathic effects within 2 days after inoculation (11). A549 (human lung carcinoma) and MRC-5 (human fetal lung fibroblast) cell lines show cytopathic effects by the third day with complete development of the CPE by the seventh day. CPE was observed to be more pronounced in the A549 cells (49).

#### Middleburg Virus

The virus has been successfully grown in BHK-21, Vero and *Aedes albopictus* C6/36 mosquito cells. The virus replicates in each of the three cell lines and consistently lysed both the BHK-21 and Vero cells within 18 hours post-inoculation. It does not lyse the mosquito cells. In BHK-21 or Vero cells, virus titers in the supernatant can rise to approximately  $10^8$  TCID<sub>50</sub> ml<sup>-1</sup> at 18 hours post-infection. The plaques produced are 3-4 mm in diameter (3).

#### Ndumu Virus

The virus has been successfully grown in Vero cells.

#### Semliki Forest Virus

Semliki Forest Virus (SFV) can grow well in many types of cell lines. Within HEp-2 (human epidermoid cancer cell line) it produces cytopathology within 24 hours, with cell destruction by 48 hours, and by 72 hours most cells have detached from surface (18). It can also grow well in C6/36, a clonal line from *Aedes albopictus*, and Chinese hamster ovary (CHO) cells. It grows well and to high titer in BHK-21 cells where virus titers can be determined by plaque assay. Chick embryo cells as well as inoculation into mouse brains are also used.

#### Western Equine Encephalitis



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Historically, isolation was done in suckling mouse intracerebral inoculations. Lately, three cell lines have been used to grow this virus; Vero cells, duck embryo fibroblasts (DEF) and Aedes albopictus C6/36 cells. Vero and DEF cultures are maintained at 37°C, whereas C6/36 cultures are held at 27°C. Titration on Vero cells can be used to estimate viral titer using standard Vero cell plaque assay (57).

#### Venezuelan Equine Encephalitis

Diagnosis of VEE is suspected on clinical and epidemiological grounds, but confirmed by virus isolation, serology, immunoassay, or nucleic acid assays. VEE viruses, like other alphaviruses, replicates readily in various cell culture systems and laboratory animal hosts. The cell lines of choice vary among laboratories, but include primary chick and duck embryo fibroblasts, VERO (African green monkey kidney) cells, fetal hamster (BHK-21) and guinea pig kidney cells, fetal guinea pig heart cells, and mouse fibroblast (L) cells. After infection, cytopathic changes occur rapidly, within 24 hours. The VEE viruses readily form plaques under an overlay (agar or gum tragacanth). Plaque morphology in vero cells under agar can be used to differentiate epizootic variants from sylvatic variants and subtype (28, 53).

There are six subtypes of VEE, numbered I through VI. Subtype I has five serotypes (A/B, C, D, E and F). VEE IABC group is pathogenic for horses and have been responsible for massive epizootics with extensive human infection and the focus of most VEE attention.

A variety of serological tests are applicable, including serology for IgM and/or IgG, using ELISA, indirect FA, hemagglutination inhibition, or complement-fixation. Both neutralizing and IgG antibody in paired sera or VEE-specific IgM present in a single serum sample indicate recent infection. Isolates can also be identified and characterized by VEE virus plaque reduction neutralization, immunofluorescence or nucleic acid testing. For persons without prior known exposure to VEE complex viruses, a presumptive diagnosis may be made by identifying IgM antibody in a single serum sample taken 5-7 days after onset of illness (28).

Virus isolation may be made from serum, cerebrospinal fluid, and in some cases throat or nasal swab specimens. Virus isolation may be performed from specimens collected in the first 3 days of illness by inoculation of cell cultures or suckling mice. Samples suitable for performing indirect diagnostic tests include acute and convalescent sera and cerebrospinal fluid. Viremia during the acute phase of the illness (but not during encephalitis) is generally high enough to allow detection by antigen-capture ELISA or other immunoassay.

#### **Immunoassay Tests.**

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

#### **Direct agent immunoassays.**

Immunofluorescent antibody tests (IFA): Direct or indirect immunofluorescent antibody tests using polyclonal or monoclonal antibodies can provide a rapid and simple means of virus identification, but because a complete battery of reagents is not yet available, this method is only used for the identification of certain viruses at present. Immunofluorescent antibody tests have been applied to direct detection of viral antigen in clinical specimens (17, 35).

Enzyme-linked immunosorbent assay (ELISA): Several ELISAs for the detection of a number of alphaviruses have been developed. ELISAs for Barmah Forest (30), Highlands J, eastern equine encephalomyelitis (23, 24), Ross River virus (30), and Venezuelan equine encephalitis (14, 21) have all been reported in the literature. Genus specific ELISAs are useful in screening where specific assays are not available (22)

Electrochemiluminescence (ECL): An ECL assay, based on the legacy M1M system, was developed for Venezuelan equine encephalitis virus with a limit of detection (LOD) of  $2.5\text{-}5 \times 10^6$  pfu/ml but some crossreactivity was seen with strains of *S. aureus*, which is common for some immunoassays, and does not work well on rabbit or dog sera. The detector and capture antibodies are a combination of two different monoclonal antibodies. Inclusivity was 100% for subtype I VEE viruses, however it was only 40% for the other subtypes, and may not detect the entire rarer enzootic strains specific to certain areas.

Lateral flow immunochromatography (LFT): Hand-held assays (HHA), "Smart tickets" have been produced by DOD and at least one commercial company that provide some limited usefulness. The DoD HHA is limited to subtype I VEE viruses. The commercial company is producing LFTs to Chikungunya virus. The DoD HHA is good for operational confirmation from automated detectors, but if evaluation is negative and that does not match operational intelligence, additional testing should be done. The same can be said for the usefulness of the commercial Chikungunya virus assay; negatives may not be negative and confirmation is probably required of the positives (1, 56).

### Serological testing.

As with other viruses, when culture or direct detection of the agent is not available, serologic procedures are still often used. Hemagglutination inhibition, ELISA, indirect immunofluorescent, and plaque reduction neutralization test (PRNT) tests in cell culture have all been used. Serological assays for Sindbis virus, western equine encephalitis complex virus (8), eastern equine encephalitis (9, 45), Mayaro virus antibodies (19), and Ross River (12, 36) have all been reported. Similar to ELISAs, where specific viral assays are not available, genus specific assays will provide some information for viral diagnostics (10, 52). Several commercial assays to the common alpha viruses (ie; chikungunya, eastern and western equine encephalitis) are available (28). Commercial assays, however, have been reported to have poor diagnostic accuracy, especially those for chikungunya virus (4, 56).

The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

### Nucleic Acid Detection Tests.

Several nucleic acid techniques have been developed for specific alpha virus detection with nucleic acid amplification by polymerase chain reaction (PCR) becoming widely used as a presumptive identification method. Real-time reverse transcriptase PCR. Barmah Forest (26, 30), chikungunya (25, 34, 38, 40, 42, 44), eastern equine encephalitis (2, 27, 32), Ross River (46, 47, 51), Sindbis (30, 48), Venezuelan equine encephalitis (6, 41, 43), and western equine encephalitis (5, 29, 31, 33) have all been described. Unlike ELISA and serology, genus specific detection of alphaviruses (39) will probably not be diagnostically useful, even as a screening method.

DoD assays have been developed, and have some validation data, for Eastern Equine Encephalitis, Western Equine Encephalitis, Western Equine Encephalitis, Chikungunya, O'nyong-nyong, and Sindbus exist. While they are not routinely deployed, they are available to support deployments if necessary or as reachback reference work at USAMRIID.

### Other Diagnostic Methods.

#### Animal inoculation

Suckling mice have been used as laboratory animals for amplifying virus in diagnostic specimens and from field-collected mosquitoes, ticks, and animal tissues. They are inoculated intracranially with clarified suspensions of specimens (28).

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# Arenaviruses: Family *Arenaviridae*

## OVERVIEW

Arenaviruses belong to the family *Arenaviridae* and are composed of largely rodent-borne viruses which are divided serologically, phylogenetically, and geographically into two major complexes, the Old World Complex (Africa, Europe, and Asia) and the New World Complex (North and South America).

The Old World Complex consists of Lassa Fever and Lujo Viruses. The New World Complex is larger and consists of Tacaribe, Pichinde, Junin, Machupo, Sabia and Guanarito viruses. Specific rodents are the principal hosts of the arenaviruses. Humans are most frequently infected through contact with infected rodent excreta, commonly via inhalation of dust or aerosolized virus-containing materials, or ingestion of contaminated foods; however, transmission may also occur through person-to-person contact with virus in the blood, tissue, secretions, or excretions of an individual infected with the virus. At least 10 arenaviruses are associated with human disease, of which six- Lassa, Lujo, Junin, Machupo, Guanarito and Sabia – are known to cause severe hemorrhagic fever in Africa, Argentina, Bolivia, Venezuela and Brazil. Arenaviruses are potential biological weapons due to their ease of dissemination, person-to-person transmissibility, and potential to cause widespread illness and death. Lassa, Junin and Machupo viruses were researched by both the US and the Soviet Union chemical and biological weapons programs as a potential biological weapon before being shut down.

## Taxonomy.

Arenaviruses have a segmented RNA genome that consists of two single-stranded ambisense RNAs and are divided into two serogroups, which differ genetically and by geographical distribution (1, 3, 5, 15, 16):

- LCMV-Lassa virus (Old World) complex - found in the Eastern Hemisphere in places such as Europe, Asia, and Africa.
- Tacaribe virus (New World) complex - found in the Western Hemisphere, in places such as Argentina, Bolivia, Venezuela, Brazil, and the United States,
  - Lymphocytic choriomeningitis (LCM) virus is the only Arenavirus to exist in both areas but is classified as an Old World virus.

Arenaviruses were formerly all placed in the genus *Arenavirus*, but were recently (2014) reclassified into the genera *Mammarenavirus* for those with mammalian hosts and *Reptarenavirus* for those infecting snakes.

## Epidemiology and Endemic Area

Rodents become chronically infected with arenaviruses, and can shed these viruses for a lifetime, hence the distribution of arenaviruses follow the distribution of their natural hosts.

Virus	Group*	Rodent Host (s)	Epidemiology and Endemic Area	Human disease
<b>Amapari virus</b>	NW	<i>Neacomys guianae</i> (Guiana bristly mouse)	Brazil	None detected so far
<b>Chapare virus</b>	NW	unknown	Samuzabeti, Chapare Province, Bolivia	None detected so far
<b>Flexal virus</b>	NW	<i>Oryzomys</i> spp. (rice rats)	Brazil	Yes
<b>Guanarito</b>	NW	<i>Zygodontomys brevicauda</i> (short-tailed cane mouse)	Venezuela	None detected so far
<b>Junin virus</b>	NW	<i>Calomys musculus</i> (drylands vesper mouse)	Argentina	Yes
<b>Latino virus</b>	NW	<i>Calomys callosus</i> (large vesper mouse)	Bolivia	None detected so far
<b>Machupo virus</b>	NW	<i>Calomys callosus</i> (large vesper mouse)	Bolivia	Yes
<b>Oliveros virus</b>	NW	<i>Bolomys obscuris</i> (Dark bolo mouse)	Argentina	None detected so far



<b>Paraná virus</b>	NW	<i>Oryzomys buccinatus</i> (Paraguayan rice rat)	Paraguay	None detected so far
<b>Pichinde virus</b>	NW	<i>Oryzomys albigularis</i> (Tomes's rice rat)	Colombia	None detected so far
<b>Piritat virus</b>	NW	<i>Sigmodon alstoni</i>	Venezuela	Yes
<b>Sabiá virus</b>	NW	unknown	Brazil	Yes
<b>Tacaribe virus</b>	NW	<i>Artibeus</i> sp. bats	Trinidad, West Indies	One suspected; moderately symptomatic
<b>Tamiami virus</b>	NW	<i>Sigmodon hispidus</i> (hispid cotton rat)	US; Florida	Occasional mild disease
<b>Whitewater Arroyo</b>	NW	<i>Neotoma</i> spp.	US; New Mexico	Yes
<b>Cupixi</b>	OW	<i>Oryzomys gaeldi</i> (rice rat)	Brazil	None detected so far
<b>Gbagroube virus</b>	OW	<i>Mus (Nannomys) setulosus</i> (African pigmy mouse)	Ivory Coast	None detected so far
<b>Ippy virus</b>	OW	<i>Arvicanthis</i> sp. (grass rats)	Central African Republic	None detected so far
<b>Junin</b>	OW	<i>Calomys musculinus</i> (dry lands vesper mouse or corn mouse)		Yes
<b>Kodoko virus</b>	OW	<i>Mus (Nannomys) minutoides</i> (savannah pygmy mouse)	Guinea	None detected so far
<b>Lassa Fever</b>	OW	<i>Mastomys</i> sp. (multimammate rat)		Yes
<b>Lemniscomys</b>	OW	<i>Lemniscomys rosalia</i> (multimammate rat)	Tanzania	None detected so far
<b>Lujo</b>	OW	unknown	Zambia, Republic of South Africa	None detected so far
<b>Luna virus</b>	OW	<i>Mastomys natalensis</i> (multimammate rat)	Zambia	
<b>Lymphocytic choriomeningitis virus (LCM)</b>	OW	<i>Mus musculus</i> (house mouse)	Americas, Europe	Yes but not severe
<b>Machupo</b>	OW	<i>Calomys callosus</i> (large vesper mouse)	Bolivia	Yes
<b>Menekre virus</b>	OW	<i>Hylomyscus</i> sp. (African wood mouse)	Ivory Coast	None detected so far
<b>Merino Walk virus</b>	OW	<i>Myotomys unisulcatus</i> (Busk Karoo rat)	Republic of South Africa	None detected so far
<b>Mobala virus</b>	OW	<i>Praomys</i> sp. (soft-furred mouse)	Central African Republic	None detected so far
<b>Mopeia virus</b>	OW	<i>Mastomys (Praomys) natalensis</i> (natal multimammate rat)		None detected so far
<b>Morogoro virus</b>	OW	<i>Mastomys natalensis</i> (multimammate rat)	Tanzania	None detected so far
<b>Mus minutoides</b>	OW	<i>Mus minutoides</i> (savannah pygmy mouse)	Tanzania	None detected so far
<b>Sabia</b>	OW	Unknown	Brazil	Yes
*NW=New World OW=Old World				

## LABORATORY DIAGNOSTICS

Biosafety Information.

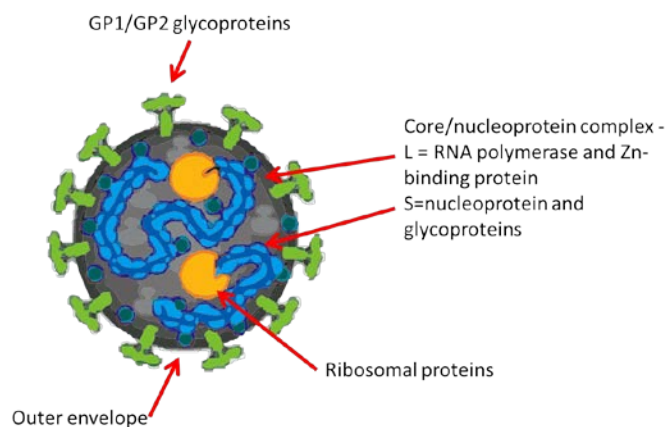
UNCLASSIFIED

NAME	RECOMMENDED BIOSAFETY LEVEL	BASIS OF RATING
<b>Sabia</b>	4	Placed in BSL-4 based on the close antigenic relationship with a known BSL-4 agent plus insufficient experience.
<b>Guanarito</b>	4	Placed in BSL-4 based on the close antigenic relationship with a known BSL-4 agent plus insufficient experience.
<b>Junin</b>	4	Level assigned to prototype or wild-type virus.
<b>Lassa</b>	4	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
<b>Machupo</b>	4	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations

## Diagnostic Information.

### General.

Arenaviruses have a segmented negative-strand RNA viruses consisting of S and L genomic segments. The genes are oriented in both negative and positive senses on the two RNA segments, a coding strategy which is called ambisense (part of the nucleotide sequence is of positive-sense, part is of negative-sense) RNAs. The two RNA segments are denoted "small" and "large" and code for four viral proteins in a unique ambisense coding strategy. Each RNA segment codes for two viral proteins in opposite orientation such that the negative-sense RNA genome serves as the template for transcription of a single mRNA and the positive-sense copy of the RNA genome templates a second mRNA.



In outbreaks of VHFs, infections are confirmed by various laboratory diagnostic methods. Virus detection is performed by virus isolation, reverse transcription-polymerase chain reaction (RT-PCR), and antigen-capture immunoassay. Serological detection of specific IgM and IgG antibodies is difficult due to cross reactions between species. An IgM-specific immunoassay is suitable for detecting recent infection, but the relevance of IgM testing for acute VHF depends on the virus and the duration of illness; specific IgM is not often present in the very early stage of illness, and patients who die of VHF often fail to seroconvert at all.

The viruses can be found in many rodent secretions and excretions including urine, saliva and respiratory secretions. Humans can be infected when arenaviruses contact mucous membranes or broken skin. This may occur during direct contact with rodents, including bites, or by indirect contact such as inhalation of aerosolized excretions and secretions, or contact with contaminated food. Rodent urine is often thought to be the source of the virus. Agricultural activities have been linked to infections with some arenaviruses when workers are exposed to aerosolized virus from harvesting machinery. Aerosols generated during virus manipulation are often implicated in laboratories. Person-to-person transmission of arenaviruses can occur in blood, urine, feces, saliva, vomit, semen and other secretions or excretions.

### Culture.

Arenaviruses can be propagated in vero cells or other cells based on the virus but optimal recovery of virus from a patient differs based on the stage of illness. Some viruses, like Lassa, Junin, and Machupo, all have differences in the best sample, dependent on the time of symptom onset; from acute illness to upwards of months after onset (for urine samples) arenaviruses (16).

### Lassa fever

The virus grows to high titers in vero, L and swine kidney cells lines as well as in diploid human cells and primary human embryo kidney cells. Infected cells can be demonstrated by

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immunofluorescence. In BHK-21, CV-1, HeLa, FL, Hep-2 and dog kidney cell lines, the virus reproduced to lower titers. The virus plaques under agar overlay only in CV-1 and Vero cells.

Cytopathic effect was observed in Indian muntjac, 8625 and Vero cells. In VSW, BHK, PtK 2, PK 15 and RD, Lassa could only be detected by indirect fluorescence antibody test (IFA). Growth in these cell lines can take anywhere from 3-14 days depending on the cell line utilized.

Recently, it has also been shown that Lassa virus can also enter polarized Madin-Darby canine kidney (MDCK) cells.

#### Lujo virus

The virus can be harvested from cell supernatants 4 days post infection in Vero-E6 cells. Lujo virus does not produce cytopathic effect in Vero-E6 cells and propagation are determined by IFA 2 to 3 days post infection with Lujo virus-specific antisera.

#### Junin virus

Junin virus grows in A549, Vero, 293AD, 293T, NMuMG, NR-9456, BHK-21 and HeLa cells. The virus can be harvested from most cells supernatant 2 days post infection, with the exception of BHK-21 cells taking 6 days, and virus titers can be determined by plaque assay in Vero cells. Titers of Junin passaged in Vero cell is between  $10^6$  and  $10^7$  PFU/ml and CPE is noticeable within 4 to 6 days.

#### Machupo virus

Machupo virus has been propagated in a number of cell lines, but peak infectivity titers seldom exceed  $10^6$ /ml. In both animals and man, highest concentrations of virus are found in lymphoid tissues. With hope of an in vitro parallel, two lines of human lymphoblastoid cells were inoculated with a human spleen isolate of Machupo virus which had been passaged twice in suckling hamster brain. The Raji cell line was derived from cells from a Burkitt lymphoma patient and PGLC 33H, from the blood of a patient with infectious mononucleosis. At 7 days post inoculation, titers of  $10^{10}$  plaque-forming unites (PFU)/ml were obtained by plaque assay in Vero cells.

#### Guanarito virus

The Guanarito virus, was isolated in Vero cells, and after 7 to 10 days, spot slides of the culture were examined for viral antigen by indirect fluorescent antibody test and by plaque reduction neutralization tests, with polyclonal hyperimmune mouse ascitic fluid made against Guanarito virus.

### Immunoassay Tests.

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

#### Direct agent immunoassays.

Lateral flow immunochromatography assays (LFIA/LFT/LFA), hand-held assays (HHA), "Smart tickets": Lateral flow assays are not widely available for most of the arenaviruses. DoD has not invested in the development and only one commercial company is known to have produced some assays for specific arenaviruses; particularly Lassa and Junin (4).

Immunofluorescent antibody tests (IFA): Direct or indirect immunofluorescent antibody tests using polyclonal or monoclonal antibodies can provide a rapid and simple means of virus identification, but because a complete battery of reagents is not yet available, this method is only used for the identification of some of the arenaviruses at present (7). Many are used in combination with culture for culture confirmation and assays for Lassa and LCM are usually considered definitive (15).

Enzyme-linked immunosorbent assay (ELISA): Several ELISA assays for particular arenaviruses have been developed for direct detection of viruses (14, 15), but many are not widely available for use outside a particular laboratory.

Serological testing. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

Indirect fluorescent antibody (IFA): IFAs were the principle method for many years and virus specific assays were widely developed by many investigators (15, 16). IFA is being replaced by ELISA assays but are still available in some laboratories.

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Enzyme-linked immunosorbent assay (ELISA): ELISA assays for antibodies to arenaviruses are the principle serological method (10, 11, 15, 16). ELISA for Lassa virus infections are the most common method being employed in Africa to monitor outbreaks (2, 8).

**Nucleic Acid Detection Tests.**

Nucleic acid amplification assays have been developed for specific arenaviruses identification and are rapidly becoming the method of choice for direct agent detection and characterization. Real-time PCR methods for most of the arenaviruses have been developed (6, 18). Validation and routine deployment are often lagging due to considerable genetic variation between the viruses the rare occurrence of many of the viruses. Assays for Lassa, Junin, and Machupo are the most militarily relevant and are well published in the literature (2, 6, 9, 13, 12).

DoD assays have been developed, and have some validation data, for Guanarito, Junin Rumero, Lassa Josiah, Lassa Macenta, Lassa Mobala (Acar), Lassa Mozambique (Mopeia), Lassa Pinneo, Lassa Weller, Machupo (pan), Machupo Carvallo, Machupo Mallele Pichinde, and Sabia exist. While they are not routinely deployed, they are available to support deployments if necessary or as reachback reference work at USAMRIID (17).

**Other Diagnostic Methods.**

Electron microscopy (EM): Using electron microscopy with negative staining, visualization of the electron-dense particles within a club shaped viral envelope is characteristic, but the virions are pleomorphic making the diagnosis more difficult than for other viruses (15).

**Animal inoculation**

Suckling mice, guinea-pigs, and hamsters have been used as laboratory animals for isolating arenaviruses (16).

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# Bunyaviridae Family

## OVERVIEW

*Bunyaviridae* are vector-borne viruses. Most of the viruses are transmitted by arthropod vectors (and hence are known as arboviruses, from *arthropod borne*). In general, bunyaviruses are transmitted by mosquitoes or midges, naireoviruses by ticks and phleboviruses by sandflies or ticks. Hantaviruses do not have arthropod vectors but are maintained in nature as persistent infections of rodents (hence the term *robovirus*, from *rodent-boorne*) and are transmitted to humans via aerosolized infectious rodent secretions. Several viruses of the *Bunyaviridae* virus family can produce mild to severe disease in human, in animals, and sometimes in both. Some of these viruses are serious human pathogens, which is the basis for requiring high containment when handling them. For example, Crimean-Congo hemorrhagic fever virus is associated with high levels of morbidity and mortality consequently handling of these viruses must occur within a Biosafety level 4 laboratory.

## ORGANISM INFORMATION

### Taxonomy.

The *Bunyaviridae* family comprises more than 300 members grouped into five genera: *Othrobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* (Tospoviruses infect only plants) (12, 36). The viruses are negative-sensed, single-stranded RNA enveloped viruses.

**Hantavirus; type species: Hantaan virus**

The name hantavirus is derived from the Hantan River area in South Korea, for which Hantaan virus is named. It was isolated in 1976 after a 25 year search for the etiologic agent that caused more than 3000 troops to become ill during the Korean War. The Sin Nombre hantavirus, first recognized in 1993, is one of several New World hantaviruses circulating in the US. Old World hantaviruses, found in Asia, can cause Hemorrhagic Fever with Renal Syndrome (HFRS). Regions especially affected by hemorrhagic fever with renal syndrome include China, the Korean Peninsula, Russia (Hantaan, Puumala and Seoul viruses), and northern and western Europe (Puumala and Dobrava virus). Regions with the highest incidences of hantavirus pulmonary syndrome (HPS) include Patagonia Argentina, Chile, Brazil, the United States, Canada, and Panama.

**Nairovirus: Crimean-Congo Hemorrhagic fever virus**

Nairovirus got its name from the Nairobi sheep disease that affects the gastrointestinal tracts of sheep and goats. Viruses in this genus are tick-borne viruses that can have human or animal hosts.

**Orthobunyavirus; type species: Bunyamwera virus**

There are currently more than 170 viruses recognised in this genus assembled into 48 species and 19 serogroups. The genus is most diverse in Africa, Australia and Oceania, but occurs almost world-wide. In the US, the California serogroup contain viruses such as La Crosse and California encephalitis viruses.

**Phlebovirus; type species: Rift Valley fever virus**

Rift Valley fever virus was first identified in 1931 in the Rift Valley of Kenya. Since then, outbreaks have been reported in sub-Saharan and North Africa. In 2000, cases were confirmed in Saudi Arabia and Yemen, making the first occurrence outside of Africa.

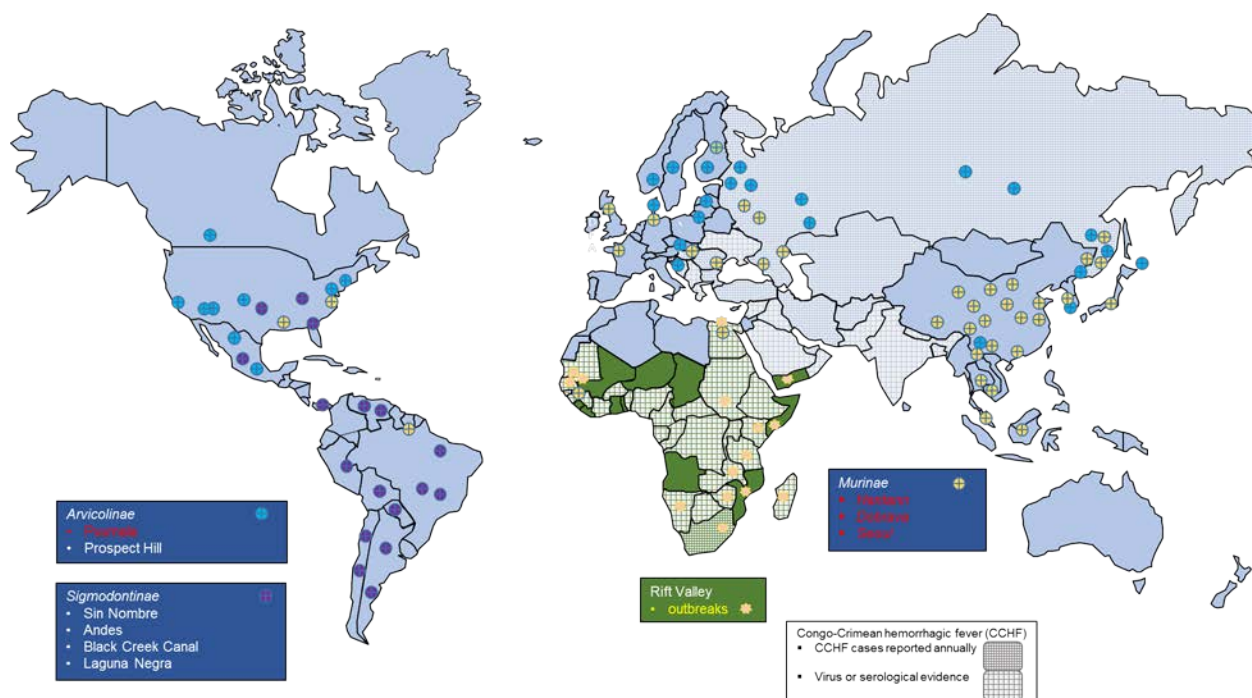
**Tospovirus; type species: Tomato spotted wilt virus**

The genus takes its name from the discovery of Tomato spotted wilt virus (TSWV) in Australia in 1915. Not further discussed.

## Epidemiology and Endemic Area

Viruses in this diverse family cause disease in humans, domesticated animals, and plants; the latter being beyond the scope of this manual. With regard to human disease, four types of illness are associated with the Bunyaviridae: fever, encephalitis, hemorrhagic fever, and a fatal respiratory syndrome, with hemorrhagic fever being of the most concern from the biothreat perspective.





Genus and Virus	Vectors and Hosts	Epidemiology and Endemic Area
<b>Hantavirus/Hantavirus</b>	rodent secretions rodent hosts are virus specific and coevolutionary	Oldworld Hantaan is found only in Asia, Dobrava is Balkans and Europe Seoul is Worldwide Puumala is Northern Europe  New World Hantaan: Prospect Hill is US Sin Nombre and Black Creek Canal in North America Andes in Argentina and Chile Laguna Negra in Brazil and Paraguay Choclo in Panama. (38)
<b>Nairovirus/Crimean Congo hemorrhagic fever virus (CCHFV)</b>	Over 30 different tick species from the genera <i>Haemaphysalis</i> and <i>Hyalomma</i> . Ruminants, birds, rodents and hares act as the amplifying hosts.	CCHF is endemic in all of Africa, the Balkans, Middle East and in Asia south of the 50° parallel north. Case fatality ratio (10-40%) (11)
<b>Orthobunyavirus/ La Crosse virus &amp; California encephalitis virus</b>	Most orthobunyavirus species are transmitted by gnats but in the US the <i>Aedes triseriatus</i> mosquito in Appalachian and Midwestern regions and <i>Aedes albopictus</i> in the South Eastern regions are vectors also. Chipmunks and gray squirrels serve as amplifying hosts.	The genus is most diverse in Africa, Australia and Oceania, but occurs almost world-wide. In the US, La Crosse virus in Appalachian, midwestern regions, and the south east, and California encephalitis virus in California and the midwest are the two most frequently encountered with low with less than 1% mortality.
<b>Phlebovirus/ Rift Valley fever</b>	Spread by <i>Aedes</i> or <i>Culex</i> genera. Virus is amplified in ruminant hosts with sheep and cattle being primary.	Rift Valley region of Kenya is the origination but outbreaks in Egypt (1977), Western Africa (1988) and the Arabian Peninsula (2000) have demonstrated its increased spread, consistent with the vectors presence. Typically less than 1% cases mortality, but Egypt in 1977, with an estimated 200,000 cases, saw 598.

## LABORATORY DIAGNOSTICS

### Biosafety Information.

Laboratory work with *Bunyaviridae* is dependent on the specific strain of virus being worked with. Biosafety ranges from BSL2 to BSL4.

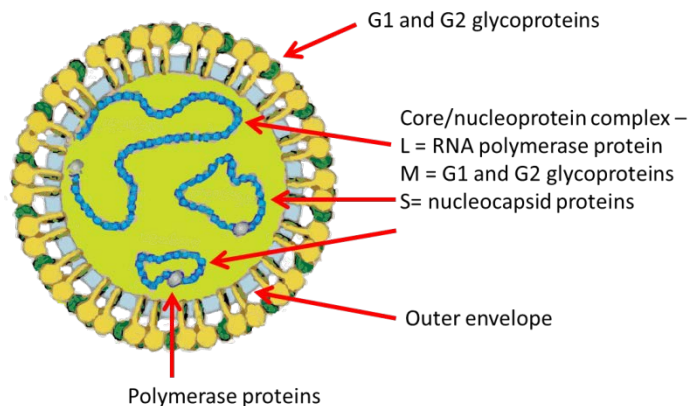
NAME	TAXONOMIC STATUS (FAMILY OR GENUS)	RECOMMENDED BIOSAFETY LEVEL	BASIS OF RATING
<b>Rift Valley Fever</b>	<i>Phlebovirus</i>	3 agricultural importance by the USDA	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
<b>Congo-Crimean Hemorrhagic Fever</b>	<i>Nairovirus</i>	4	Level assigned to prototype or wild-type virus.
<b>La Crosse</b>	<i>Orthobunyavirus</i>	2	Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
<b>Andes</b>	<i>Hantavirus</i>	3 - Containment requirements will vary based on virus concentration, animal species, or virus type.	Insufficient experience with virus in laboratory facilities with low biocontainment
<b>Dobrava-Belgrade</b>	<i>Hantavirus</i>		Insufficient experience with virus in laboratory facilities with low biocontainment
<b>Hantaan</b>	<i>Hantavirus</i>		Subcommittee on Arbovirus Laboratory Safety (SALS) first provided biosafety recommendations
<b>Seoul</b>	<i>Hantavirus</i>		Insufficient experience with virus in laboratory facilities with low biocontainment
<b>Sin Nombre</b>	<i>Hantavirus</i>		Insufficient experience with virus in laboratory facilities with low biocontainment

### Diagnostic Information.

#### General.

Bunyaviruses are negative-stranded, enveloped RNA viruses. Bunyaviridae have a three part genome that consists of a large (L), medium (M), and small (S) RNA segment. The L segment encodes the RNA dependent RNA-polymerase for viral RNA replication and mRNA synthesis. The M segment encodes the viral glycoproteins; the projects from the viral membrane that is used for attaching to and entering the host cell. The S segment encodes the nucleocapsid protein. Total genome size ranges from 10.5 to 22.7 kbp.

Bunyaviruses, like most viral diseases, are best diagnosed by isolating the virus, detecting RNA by nucleic acid amplification, or by showing a fourfold or greater rise in antibody titer between acute- and convalescent-phase sera. The virus can be isolated from blood (or from brain, liver, and other organs postmortem) during the viremic phase, but not usually after the third day of fever. It is propagated in baby mice or mosquitoes or in vertebrate or invertebrate tissue cultures (6, 5, 26). Fluorescent antibody tests and the enzyme-linked immunosorbent assay (ELISA) are often group reactive but the neutralization inhibition tests are typically type specific. Assessments of IgM may be especially useful in establishing an early diagnosis. Once isolated, virus is identified by the same tests with a reference immune serum.



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Bunyavirus diseases usually are restricted to focal geographic areas because of the limited distribution of their vectors and vertebrate hosts. Awareness of their geographic distribution, seasonality, and clinical syndrome may help in establishing a diagnosis. For instance, hemorrhagic fever with renal syndrome should be strongly suspected in a person in Europe or Asia who has fever, proteinuria, thrombocytopenia, and elevated blood urea nitrogen, especially if the patient has been exposed to rodents. Definitive diagnosis, however, can be made only by laboratory tests.

#### Culture.

Bunyaviruses can be propagated in vero cells or other cells based on the virus but optimal recovery of virus from a patient differs based on the stage of illness.

#### Crimean-Congo hemorrhagic fever virus

Vero cells and chicken embryo reticulum (CER) can be used to grow CCHFV (39). CCHFV has also been reported to replicate in cell lines derived from the ticks *Hyalomma anatolicum*, *Amblyomma variegatum*, *Rhipicephalus (Boophilus) decoloratus*, *Rhipicephalus (Boophilus) microplus*, and *Ixodes ricinus* (10). None of the tick cell lines exhibited any cytopathic effect for 21 days following infection and propagation is typically detected by real-time reverse transcription-PCR (44) or indirect immunofluorescence. CCHFV is capable of producing plaques in CER cells with plaques visible with microscopy after 3 days of incubation and can be visible 24 hours later with the application of a staining overlay (13). At 4 to 5 days post inoculation, plaques are 2 to 3 mm in diameter, but longer incubation periods result in the coalescence of plaques, which then become difficult to read. Reproducible results with Vero cells were not seen. When present, plaques in Vero cells are small and indistinct, requiring incubation periods of 6 to 7 days. Vero cells and CER cells typically contain the same amount of virus when titrated by the fluorescence focus assay test (39).

#### Hantavirus

Hantaviruses are slow to adapt to growth in cell culture and only a few cell lines, Vero E-6, A549 and HEK293 are known to support growth. Vero E-6 and A549 infected cell cultures do not exhibit cytopathic effect therefore the presence of infectious virus must be established by demonstration of specific viral antigens by immunoassay or nucleic acid assays. HEK293 cells infected with Sin Nombre Hantavirus or Andes Hantavirus showed extensive CPE. Sin Nombre virus required 12-14 days to cause strong CPE in HEK293 cells (32). Plaque reduction neutralization tests (PRNTs) or focal reduction neutralization tests (FRNTs) are used to identify and serotype hantaviruses since cytopathic effect is sometimes absent in the commonly used Vero E-6 cells. With FRNT, virus is visualized in the infected foci by staining with fluorescent or enzymatically labelled antibodies and counting the spots (23, 41).

#### La Crosse Virus

Virus can be propagated in C6/36 cells with some CPE and an infectivity titer between 5.5 and 6.6 (43). Virus grows to titers around  $1 \times 10^8$  in BHK-21 cells (21). Virus can also be isolated by inoculating mosquito homogenate onto a monolayer of Vero cells. Serum dilution plaque reduction neutralization tests can be performed on Vero cells (7).

#### Rift Valley Fever Virus

A variety of cell monolayers including Vero, BHK, mosquito cell lines, chicken embryo reticulum (CER) and primary kidney or testis cells of calves and lambs may be inoculated. After 5-6 days the virus induces a cytopathic effect characterized by slight rounding of cells followed by destruction of the whole cell sheet within 12-24 hours (14).

#### Immunoassay Tests.

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

#### Direct agent immunoassays.

Immunoassays for direct detection of most of the bunyaviruses are not widely available. DoD has not invested deeply in the development and only very little, if at all, is mentioned in the literature. The DOD does produce a lateral flow assay for Rift Valley fever virus. Immunohistochemistry and indirect fluorescent antibody (IFA) assays for use in identification and characterization of particular viruses seem to be the extent of direct detection methods.

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Serological testing. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

Lateral flow immunochromatography assays (LFIA/LFT/LFA), hand-held assays (HHA), "Smart tickets": Lateral flow assays for serological identification of antibodies to hantavirus (4, 25) have been reported.

Indirect fluorescent antibody (IFA): IFAs were the principle method for many years and virus specific assays were developed by some investigators (1, 20, 28, 30, 33, 46) for antibodies to bunyaviruses. Commercial IFAs for hanta, Rift Valley, CCHF, and others, particularly from European sources (some with US distribution), are available. IFA is being replaced by ELISA assays but are still available in some laboratories.

Enzyme-linked immunosorbent assay (ELISA): ELISA assays for antibodies to bunyaviruses is the principle serological method (22, 26). Serological ELISA have all been developed for the main viruses of concern in the Bunyaviridae family; hantavirus (24, 29, 40, 45), Rift Valley (27, 35), and CCHF (3, 15, 34, 37, 44). Like the IFAs, commercial ELISAs for hanta, Rift Valley, CCHF, and others, particularly from European sources (some with US distribution) are also available, but none are FDA approved.

Plaque reduction neutralization test (PRNT): PRNT is used for detecting and quantifying many bunyaviruses antibody titers, especially for those that can grow in lytic cell lines, like CCHF (26).

### **Nucleic Acid Detection Tests.**

Several nucleic acid techniques have been developed for specific bunyavirus identification, with nucleic acid amplification by polymerase chain reaction (PCR) becoming more widely available. Real-time PCR methods based on the S, M, and L gene segments have been developed that appear to be specific for the various bunyavirus genuses (9). There are a number of assays available for the major viruses like Rift Valley Fever (3, 16, 17, 19), Congo-Crimean Hemorrhagic Fever (8, 17, 18) and Hantavirus (2, 19, 22, 31).

DoD assays have been developed, and have some validation data, for CCHFV, Dengue, Hantavirus Andes, Hantavirus Hantaan, Hantavirus Puumala, Hantavirus Seoul, Hantavirus Sin Nombre; Hantavirus New York, Hantavirus Dobrava, Punta Toro virus, and Rift Valley Fever exist. While they are not routinely deployed, they are available to support deployments if necessary or as reachback reference work at USAMRIID (42).

### **Other Diagnostic Methods.**

#### **Animal inoculation**

Suckling mice are occasionally used as laboratory animals for isolating bunyaviruses when viral amplification is needed and cell cultures are not working (9).

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# Filoviridae Family

## OVERVIEW

Filoviridae consist of several related viruses that form filamentous infectious viruses. Two members of the family that are commonly known are Ebola virus and Marburg virus. Both viruses, and some of their lesser known relatives, cause severe disease in humans and nonhuman primates in the form of viral hemorrhagic fevers. The filoviruses awakened international attention in 2014 as a result of the west African outbreak that infected almost 30,000 and resulted in over 11,000 deaths in 10 countries. Attention and fear generated by the 2014 outbreaks of filoviruses rose worldwide. Although they are not considered naturally transmitted by aerosol, they are highly infectious as respirable particles under laboratory conditions (11).

## ORGANISM INFORMATION

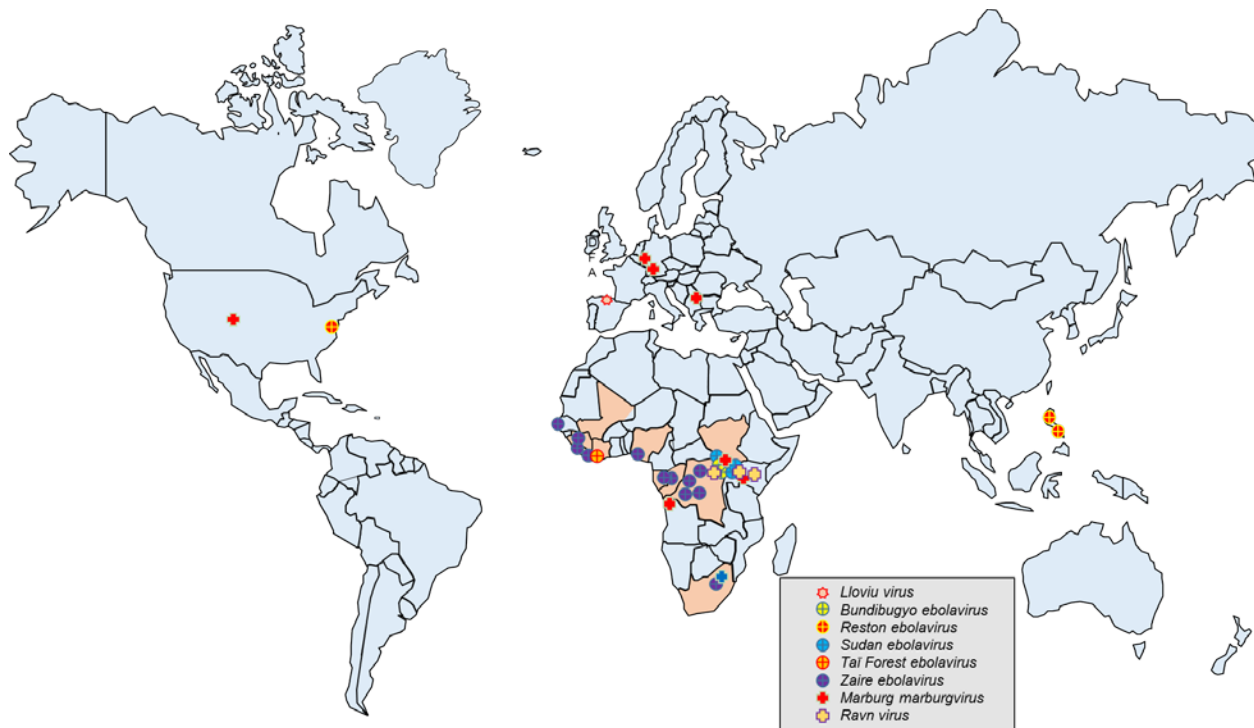
### Taxonomy.

The family currently includes the three virus genera Cuevavirus, Ebolavirus, and Marburgvirus. The viruses are single-stranded negative-sense RNA, filamentous viruses currently comprising three genera and eight species.

- Cuevavirus or Lloviu cuevavirus, consists of only Lloviu virus (LLOV), found in bats in Spain, Portugal and France; not apparently pathogenic for humans (13).
- Ebolavirus contains five species;
  - Bundibugyo ebolavirus: Bundibugyo virus (BDBV; previously BEBOV) was first identified in 2007 in the Bundibugyo District of Uganda.
  - Reston ebolavirus: Reston virus (RESTV; previously REBOV) was first identified in crab-eating macaques from Hazleton Laboratories at Reston, Virginia in 1989. Affected animals had been imported from a facility in the Philippines, which continues to be the source of other isolations.
  - Sudan ebolavirus: Sudan virus (SUDV; previously SEBOV) was first identified in Nzara, Sudan cotton factory workers (now in South Sudan) in 1976.
  - Tai Forest ebolavirus: Tai Forest virus (TAFV; previously CIEBOV) was formerly known as "Côte d'Ivoire ebolavirus." Tai Forest was first identified from chimpanzees in the Tai Forest, Côte d'Ivoire, Africa, in 1994.
  - Zaire ebolavirus: Ebola virus (EBOV; previously ZEBOV) is more common than the other species. The first outbreak took place in 1976 with the latest in West Africa in 2014-15.
- Marburgvirus or Marburg marburgvirus, consists of
  - Marburg virus (MARV); this virus was first described in 1967 during outbreaks in the German cities Marburg and Frankfurt and the Yugoslav capital Belgrade in workers were accidentally exposed to tissues of infected grivet monkeys (*Chlorocebus aethiops*).
  - Ravn virus (RAVV); the virus was first described in 1996 and name Ravn virus is derived from the name of the Danish patient from whom this virus was first isolated.

### Epidemiology and Endemic Area

The natural reservoir (or reservoirs) for filoviruses are unknown. It is commonly assumed that filoviruses are transmitted from animals to humans. While the virus can be replicated in fruit and insectivorous bats, native to the area where filoviruses are found, they have not conclusively been shown to be a source of human infections.



## LABORATORY DIAGNOSTICS

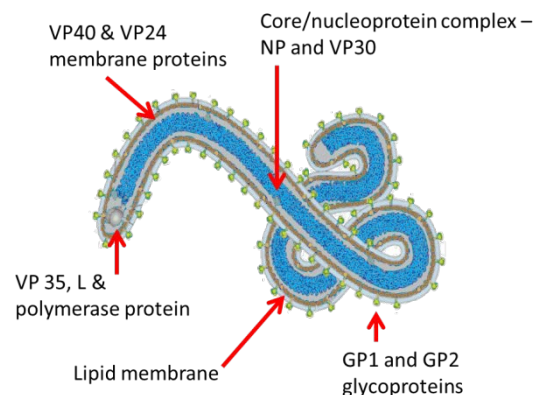
### Biosafety Information.

NAME	RECOMMENDED BIOSAFETY LEVEL
<b>Ebola (Including Reston)</b>	4
<b>Marburg</b>	4

### Diagnostic Information.

#### General.

Filoviruses are filamentous viruses that may appear in the shape of a shepherd's crook or in the shape of a "U" or a "6", and they may be branched, circular, or coiled. Filoviruses are generally 80 nm in width, and ranges from 795 to 1,086 nm in length. The filoviridae genome is a single-stranded negative-sense RNA that encodes seven structural proteins. The helical ribonucleocapsid consisting of genomic RNA wrapped around nucleoproteins (NP). Associated with the genomic RNA is an RNA-dependent RNA polymerase (L) with the polymerase cofactor (VP35) and a transcription activator (VP30). These viruses also have a lipid membrane, like most viruses, that anchor glycoproteins (GP1, 2) that project 7 to 10 nm from its surface. Under the lipid membrane is a protein structure of VP40 and VP24.



#### Culture.

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Filoviruses can be easily propagated in vero E6 cells with production of cytopathic plaques, but other cell lines also work well (5, 14).

**Immunoassay Tests.**

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

**Direct agent immunoassays.**

Indirect fluorescent antibody (IFA): IFAs are still a primary method for confirmation of cell culture growth (5, 14). Other uses of IFA for the filoviruses are not described.

Enzyme-linked immunosorbent assay (ELISA): ELISA assays for direct filoviruses detection are useful for both tissue culture confirmation as well as patient sample testing (1, 7, 14, 15). Particularly good are the monoclonal antibody based ELISAs that target the VP40 or VP30 proteins. The use of recombinant proteins for antibody production has improved the sensitivity and specificity of many assays.

Lateral flow immunochromatography assays (LFIA/LFT/LFA), hand-held assays (HHA), "Smart tickets": Lateral flow assays are not widely available for most of the filoviruses. DoD has developed a HHA for Ebola, but has not made it widely available. Due to the Ebola outbreak in 2014, several commercial companies have produced some assays for Ebola and Marburg also; some of which are FDA approved under the emergency use authorization (for as long as that lasts or until they get specific 510K approval) (1, 17).

Serological testing. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

Indirect fluorescent antibody (IFA): IFAs were the principle method for filovirus serology for many years but IFA has given way to ELISA and nucleic acid methods recently. Problems with antigen specificity has largely been overcome with wider adoption of recombinant viral proteins and have reduced the need for BSL4 facilities when testing patient samples (3, 12, 14).

Enzyme-linked immunosorbent assay (ELISA): ELISA assays for antibodies to the filoviruses have become the principle serological method (8-10). Many assays have been introduced since the 2014 Ebola outbreak, but none are FDA approved for direct patient use.

**Nucleic Acid Detection Tests.**

Several nucleic acid techniques have been developed for specific Filoviridae identification, with nucleic acid amplification by polymerase chain reaction (PCR) most widely used. Real-time PCR methods have been developed for each of the specific filovirus strains (2, 4, 14). A broad Filoviridae RT-PCR is not commonly used because of the inherent genetic differences between the various strains, though some "pan-Ebola" and "pan-Marburg" assays have been developed. Again, as a result of the 2014 West Africa Ebola outbreak, PCR assays for Ebola Zaire flooded the FDA EUA system and many will probably get official FDA sanctioning for patient testing and be commercially available.

Like the other viruses, DoD assays have been developed and have some validation data for Ebola (pan), Ebola Ivory Coast, Ebola Ivory Coast, Ebola Reston, Ebola Reston, Ebola Sudan, Ebola Sudan, Ebola Zaire, Ebola Zaire, Marburg (pan), Marburg (pan), Marburg Angola, Marburg Angola, Marburg Ci67, Marburg Musoke, and Marburg RAVN (16).

**Other Diagnostic Methods.**

Electron microscopy (EM): Using electron microscopy with negative staining or immunogold visualization of the characteristic structure is usually good for presumptive identification (6, 14). Samples from blood and urine, along with cell culture supernate, have been used.

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# Flaviviridae Family

## OVERVIEW

The Flaviviridae are a family of viruses that are primarily spread through arthropod vectors (mainly ticks and mosquitoes). The family gets its name from the Yellow Fever virus; flavus means yellow in Latin. Yellow fever was named because of its propensity to cause jaundice in victims. Currently, more than 70 flaviviruses have been reported, and many of them cause human disease. All human flaviviruses are transmitted by ticks and mosquito vectors, making the disease very difficult to eradicate. The viruses within the Flaviviridae family are associated with significant public health and cause economic impacts worldwide.

## ORGANISM INFORMATION

### Taxonomy.

The family contains four genera: Hepacivirus, Flavivirus, Pegivirus and Pestivirus. Flavivirus is the main genus of biothreat concern and current phylogenetic analysis describe more than 70 species of flaviviruses. These flaviviruses have been grouped into 14 clades, which in turn can be grouped in three clusters: the mosquito-borne cluster, the tick-borne cluster, and cluster with no specifically identified vector. All flaviviruses of human importance belong to the first two clusters; the last cluster holds a few viruses, which have been isolated from mice or bats (21, 44).

- Genus Flavivirus (type species Yellow fever virus, others include West Nile virus and Dengue Fever)—contains 67 identified human and animal viruses
- Genus Hepacivirus (type species Hepatitis C virus, also includes GB virus B)-not further discussed.
- Genus Pegivirus (includes GB virus A, GB virus C, and GB virus D) -not further discussed.
- Genus Pestivirus (type species bovine viral diarrhea virus, others include classical swine fever or hog cholera)—contains viruses infecting non-human mammals and not further discussed.

### Epidemiology and Endemic Area

Genus and Virus	Vectors and Hosts or Mode of Transmission	Epidemiology and Endemic Area
<b>Alkhurma hemorrhagic fever</b>	Tick <i>Ornithodoros savignyi</i> (sand tampans) with sheep and camels as primary hosts.	Saudi Arabia The fatality rate for humans is 30%
<b>Dengue fever</b>	<i>Aedes</i> mosquitoes, particularly <i>A. aegypti</i> but include <i>A. albopictus</i> , <i>A. polynesiensis</i> and <i>A. scutellaris</i> . Humans are the primary host of the virus, but it also circulates in nonhuman primates.	Found in tropical and sub-tropical regions around the world and is common in more than 110 countries.  The fatality rate for humans is 1–5%, and less than 1% with adequate treatment. It infects 50 to 528 million people worldwide a year, leading approximately 25,000 deaths.
<b>Japanese encephalitis</b>	Mosquito, primarily <i>Culex tritaeniorhynchus</i> and <i>Culex vishnui</i> . The vertebrate hosts are primarily pigs and wading birds (herons).	Endemic throughout most of Asia and parts of the Western Pacific region (China, Japan, South Korea, Nepal, northern Vietnam, and northern India).  Fatality rate for humans is 0.3% to 60%, dependent on the population and age. Up to 70,000 cases reported annually.
<b>Kyasanur Forest</b>	Ticks, mainly <i>Haemaphysalis spinigera</i> (forest tick) but also <i>Dermacentor</i> and <i>Ixodes</i> . The main hosts are small rodents, but bats, monkeys, and shrews may carry the virus.	Limited to Karnataka State, India and near by areas.  Fatality rate for humans is 3-5% with between 400-500 humans cases per year.



<b>Murray Valley</b>	<p>Mosquito, <i>Culex annulirostris</i> (the common banded mosquito), other <i>Culex</i> species and some <i>Aedes</i> species.</p> <p>Primary hosts are thought to be wading birds such as herons and egrets.</p>	<p>Endemic to northern Australia and Papua New Guinea.</p> <p>Fatality rate for humans is 15%–30% with long-term neurological sequelae occurring in 30%–50% of survivors with only about 40% of those infected completely recovering.</p>
<b>Omsk Hemorrhagic Fever</b>	<p>Tick <i>Dermacentor reticulatus</i> with water voles and muskrats as primary hosts.</p>	<p>Endemic in Omsk, Siberia Russia.</p> <p>Fatality rate for humans is &lt;3%</p>
<b>St. Louis Encephalitis</b>	<p>Mosquitoes, <i>Culex</i> species, including <i>Culex pipiens</i> and <i>Culex quinquefasciatus</i> in the East, <i>Culex nigripalpus</i> in Florida, and <i>Culex tarsalis</i> and members of the <i>Culex pipiens</i> complex in Western states.</p> <p>Birds, such as house sparrows, pigeons, blue jays, and robins are primarily hosts.</p>	<p>Temperate areas of the United States, primarily in the late summer or early fall. In the southern states, where the climate is milder, cases can occur year round.</p> <p>From 1964 through 2009, an average of 102 cases were reported annually (range 2-1,967).</p> <p>Fatality rate for humans is 5 to 15%. Annual cases in the US are only about 100 annually.</p>
<b>Tick-borne Encephalitis</b>	<p>Ticks of the <i>Ixodes</i> species, primarily <i>Ixodes ricinus</i> (European subtype) or <i>Ixodes persulcatus</i> (Siberian and Far Eastern subtypes).</p> <p>Ticks act as both vector and virus reservoir, and small rodents are the primary amplifying host.</p>	<p>Endemic in areas of 19 Europe countries and Asia (from eastern France to northern Japan and from northern Russia to Albania).</p> <p>Fatality rate for humans is &lt;2% in Europe but 20%–40% in Asia, Average of 8,500 cases per year.</p>
<b>West Nile</b>	<p>Various mosquito species, particularly <i>Culex pipiens</i>. Birds are a prime host reservoir but other animals are potential hosts.</p>	<p>Found in temperate and tropical regions of the world. First identified in the West Nile sub region in the East African nation of Uganda in 1937, it is considered endemic in Africa, Asia, Australia, the Middle East, Europe and the United States.</p> <p>The fatality rate for humans is 3–15%, predominately associated with older age. About 5000 cases are reported in the US annually.</p>
<b>Yellow Fever</b>	<p>There are three epidemiologically different infectious cycles, in which the virus is transmitted from mosquitoes to humans or other primates. In the "urban cycle," only the yellow fever mosquito <i>Aedes aegypti</i> is involved. In the sylvatic cycle (forest cycle or jungle cycle) <i>Aedes africanus</i> (In Africa) or <i>Haemagogus</i> and <i>Sabethes</i> genus (in South America) are the vectors. The third infectious cycle, also known as the savannah cycle, the genus <i>Aedes</i> is the vector.</p>	<p>Endemic in tropical and sub-tropical regions areas of South America and Africa.</p> <p>The fatality rate for humans is 40-100% and causes 200,000 infections and 30,000 deaths every year, with nearly 90% of these occurring in Africa.</p>
<b>Zika virus</b>	<p>Mosquitoes, principally <i>Aedes</i> species, including <i>A. africanus</i>, <i>A. coarctatus</i>, <i>A. luteocephalus</i>, <i>A. vitattus</i>, and <i>A. furcifer</i>, <i>Aedes hensilli</i>, and <i>Aedes polynesiensis</i>.</p> <p>Host is unknown.</p>	<p>Equatorial; Africa, Southeast Asia, the Pacific Islands, and northern South America.</p>

## LABORATORY DIAGNOSTICS

## Biosafety Information.

NAME	RECOMMENDED BIOSAFETY LEVEL
<b>Alkhurma hemorrhagic fever</b>	4
<b>Dengue Virus</b>	2
<b>Kyasanur Forest Disease</b>	4
<b>Omsk Hemorrhagic Fever</b>	4
<b>Yellow Fever</b>	3

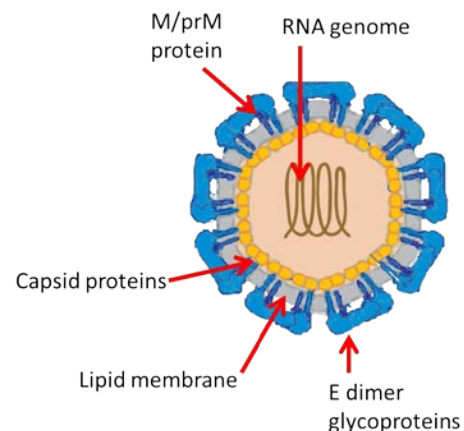
## Diagnostic Information.

## General.

Flaviviridae is an icosahedral-like, enveloped virus with a linear, single-stranded RNA genome of positive polarity, 9.6 to 12.3 kilobase in length, and about 40–60 nm in diameter.

## Culture.

Flaviviruses can be generally propagated in MK2, Vero, or other cells, based on the virus, but optimal recovery of virus from a patient differs based on the stage of illness, typically during the viremia phase; usually the first 4–5 days of the disease when symptomology is present or just starting. Some of the flaviviruses have high viral load during a patient's verimic stage and others have very low virus titers that make virus isolation low. For low blood veremias, brain or CSF may make better samples, if they can be obtained. Serum and plasma are the most common samples.

Dengue Virus

Mosquito cell line C6/36 is the method of choice for virus isolation, although other cell lines like mosquito AP61, C6/36, and AP64 or mammalian Vero, rhesus kidney epithelial cell line (LLC-MK2), or baby hamster kidney cell (BHK-21) can also be used. Sera that have been collected from suspected dengue cases in the first 3–5 days of fever (the viraemic phase) is used for virus isolation. After 1 to 4 days incubation, viral identification is performed using immunofluorescence or nucleic acid amplification assay (38, 42).

West Nile Virus

West Nile virus is a low viral load viremia virus and difficulties isolating virus from humans can be expected. The virus can be propagated in rabbit kidney (RK-13), African green monkey kidney (Vero), or pig kidney cells. Primary isolation in embryonated chicken eggs or mosquito C6/36 cell lines followed by passage in mammalian cells can also be used. More than one cell culture passage may be required to observe cytopathic effect (CPE). Confirmation of WNV isolates is achieved by indirect fluorescent antibody staining of infected cultures or nucleic acid detection methods (17).

Yellow Fever Virus

Isolation of virus from blood using mosquito cells, like *Aedes albopictus* (C6/36) or Vero, B4, MK2 or BHK21 cells incubated for 3 to 6 days. Virus produces little to no CPE in mosquito cell lines and requires confirmation by indirect fluorescent antibody staining of infected cultures or nucleic acid detection methods. Plaques fine in most mammalian cells (3).

Japanese Encephalitis (JE)

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Attempts to isolate Japanese encephalitis virus from clinical specimens are usually unsuccessful, usually because of low viral titers and the rapid production of neutralizing antibodies. Isolates may sometimes be obtained from CSF or from brain tissue (necropsy or postmortem needle biopsy). Generally, any of the mammalian cell lines, like Vero, rhesus kidney epithelial cell line (LLCMK2), and baby hamster kidney cell (BHK-21) should work for isolation (8, 17).

#### Murray Valley encephalitis virus

Culture of Murray Valley virus is not well documented but a cell line derived from larvae of *Aedes aegypti* (10), Vero, monkey kidney epithelial (MEK) (22), or chicken embryo cells have been used previously (2). Lehmann reported that chicken egg embryo cultivation was reported to be the best (22). Several cases have relied on laboratory confirmation only through serology, without culture (7, 26, 41).

#### Saint Louis Encephalitis

Saint Louis encephalitis is difficult to isolate from clinical samples and almost all isolates have come from brain tissue or CSF. Confirmatory testing involves direct detection through indirect fluorescent antibody testing or through use of specific neutralizing antibodies in viral neutralization tests such as PRNT (17).

#### Tick-borne Encephalitis/Kyasanur Forest Disease/Alkhurma hemorrhagic fever

These viruses have low, sometimes short duration, viral loads during viremia and there will be difficulties isolating virus from humans. Tick-borne encephalitis, Kyasanur Forest disease, and Alkhurma hemorrhagic fever viruses are all similar in many respects, but also not commonly encountered, making diagnosis difficult. Some of the viruses have been propagated in HeLa, swine embryo kidney, monkey kidney tissue culture, as well as possibly others (1, 23, 27, 40).

#### Immunoassay Tests.

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

#### Direct agent immunoassays.

Lateral flow immunochromatography assays (LFIA/LFT/LFA), hand-held assays (HHA), "Smart tickets": Lateral flow assays are not widely available for most of the flaviviruses. DoD has a Dengue lateral flow assay and only one commercial company is known to have produced some assays for specific arenaviruses; particularly Dengue (24, 33, 45).

Immunohistochemical (IHC) staining: Dengue antigens can be detected in tissues such as liver, spleen and lymph nodes as well as tissues from fatal cases (slides from paraffin-embedded, fresh or frozen tissues) using an enzyme and a colorimetric substrate with antibodies that target dengue-specific antigens (11, 35).

Serological testing. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays. Especially important for the flaviviruses, is determining an appropriate acute sample since the differences in antibody response for the different viruses vary.

Indirect fluorescent antibody (IFA): Serology by IFA was common use for many of the flaviviruses but is being replaced by ELISA. IFA for dengue (14) and JE (30) are two examples of IFA for flaviviruses, as well as the products of several commercial companies (17).

Lateral flow immunochromatography assays (LFIA/LFT/LFA), hand-held assays (HHA), "Smart tickets": For serological determination, at least one commercial company is known to have produced a serological LFA assay for Dengue virus (13).

Enzyme-linked immunosorbent assay (ELISA): ELISA for serological determination is in common use for many of the flaviviruses. Many of the flaviviruses have commercial ELISA kits available, but many are not FDA approved for use (17). Since these ELISA assays can cross-react with other flaviviruses, these indirect methods cannot conclusively prove virus specific infection (20, 34). The IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) has been used for diagnosis of several flaviviruses, including Dengue (4, 13, 18, 38), Kyasanur Forest Disease (32), Japanese Encephalitis (5, 12), and Tick-borne Encephalitis (29), have all been published.

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Plaque reduction neutralization assay (PRNT): PRNT and the micro-neutralization assay can be used for many of the flaviviruses, including Dengue (15, 39). Differentiation of the antibodies to a specific virus from the other flaviviruses, can be a problem since antigenic cross-reactivity in flaviviruses is common. An IgM based neutralizing response, especially from Dengue, is sometimes more specific than an IgG one.

#### Nucleic Acid Detection Tests.

Several nucleic acid techniques have been developed for specific flavivirus identification, with nucleic acid amplification by polymerase chain reaction (PCR) the most widely applied (3, 16, 19, 25, 28, 29, 31, 32, 34, 36, 37, 43). Real-time PCR methods have targeted several of the basic flavivirus genes, including those that code for the NS5 protein (one of seven non-structural proteins) or are part of the untranslated region of the genome (6, 9). While many assays have been developed, validation and FDA approval are not common. Typically, the viruses can be found in plasma or serum 2 to 7 days from disease onset but the quantity of virus in the blood and the duration for detection is very virus dependent.

#### Other Diagnostic Methods.

##### Animal inoculation

Suckling mice have been used as laboratory animals for amplifying virus in diagnostic specimens and from field-collected mosquitoes, ticks, and animal tissues. They are inoculated intracranially with clarified suspensions of specimens. For dengue, direct inoculation into competent mosquitos can be done, carefully (17).

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# Biological Toxins

Toxins are harmful substances produced by living organisms (animals, plants, microbes). A toxin's utility as an aerosol weapon is determined by its toxicity, stability, and ease of production. The bacterial toxins, such as botulinum toxins, are the most toxic substances by weight known. Less toxic compounds are thousands of times less toxic than botulinum, and have limited aerosol potential. The relationship between aerosol toxicity, quantity of toxin required, and the stability of the toxin limits an effective open-air attack in a dispersed tactical environment for many of the toxins. For example, botulinum and tetanus toxins are large-molecular-weight proteins, and are easily denatured by environmental factors (heat, desiccation, or ultraviolet light), thus posing little downwind threat.

As with all biological weapons, potential to cause incapacitation as well as lethality must be considered. Depending on the goals of an adversary, incapacitating agents may be more effective than lethal agents. Large numbers of ill patients might overwhelm the medical and evacuation infrastructure and will almost certainly create panic and disruption of the affected population. Several toxins, such as staphylococcal enterotoxin B (SEB), pose a significant incapacitating threat by causing illness at doses much lower than those required for lethality.

The three toxins most likely to be used as biothreat agents are botulinum, ricin, and SEB toxins.

# Botulinum

## OVERVIEW

Botulinum toxins are a group of seven related neurotoxins produced by the spore-forming bacillus *Clostridium botulinum* and two other *Clostridium* species. These toxins, types A through G, are the most potent neurotoxins known; ironically, they have been used therapeutically to treat medical conditions and cosmetically to treat wrinkles. The spores are ubiquitous; they germinate into vegetative bacteria that produce toxins during anaerobic growth. Industrial-scale fermentation can produce large quantities of toxin for use as a biowarfare agent. There are three epidemiologic forms of naturally occurring botulism; foodborne, infantile, and wound. Botulinum toxin can be delivered by aerosol or used to contaminate food or water supplies. When inhaled, these toxins produce a clinical picture very similar to foodborne intoxication, although the time to onset of paralytic symptoms after inhalation may actually be longer than for foodborne cases, and may vary by type and dose of toxin. The clinical syndrome produced by these toxins is known as "botulism."

Botulinum toxins have caused numerous cases of botulism when ingested in improperly prepared or canned foods. Many deaths have occurred from such incidents. It is feasible to deliver botulinum toxins as an aerosolized biological weapon, and several countries and terrorist groups have weaponized them. Evidence obtained by the United Nations in 1995 revealed that Iraq had filled and deployed over 100 munitions with nearly 10,000-19,000 liters of botulinum toxin; enough to kill the entire human population. The Aum Shinrikyo cult in Japan weaponized and attempted to disseminate botulinum toxin on multiple occasions in Tokyo before their 1995 sarin attack in the Tokyo subway.

## TOXIN CHARACTERISTICS

Botulinum toxins are the most toxic compounds, per weight of agent, requiring only one nanogram (10<sup>-9</sup> g) per kg of body weight to kill 50 percent of the animals studied. Botulinum toxin type A is 15,000 times more toxic by weight than VX and 100,000 times more toxic than sarin (GB), two of the well-known organophosphate nerve agents.

The botulinum toxin consists of two polypeptide subunits (A and B chains). The B subunit binds to receptors on nerve fibers. The toxin is taken into the nerve, where the A chain exerts its cytotoxic effect; it prevents release of acetylcholine thus blocking neuromuscular transmission (pre-synaptic inhibition).

Botulinum toxins are proteins with molecular masses of approximately 150,000 daltons. Each of the seven distinct, but related neurotoxins, A through G, is produced by a different strain of *Clostridium botulinum*. All seven types act by similar mechanisms of inhibition of presynaptic acetylcholine release. The toxins produce similar effects when inhaled or ingested, although the time course may vary depending on the route of exposure and the dose received. The most likely offensive use of botulinum toxin would be by aerosol dispersal, sabotage of food and direct water supplies, along with direct assignation are also potential uses. Large scale dissemination in water is not likely due to dilution and water treatment that would limit a lethal dose, but the terrorism potential would still cause sufficient public disruption.

The botulinum toxins are easily denatured by environmental conditions and aerosol dissemination is, at best, questionable. The toxins are detoxified in air within 12 h. Sunlight inactivates the toxins within 1-3 h. Heat destroys the toxins in 30 min at 80°C and in several min at 100°C. In water, the toxins are >99.7% inactivated by 20 min of exposure to 3 mg/L free available chlorine (military water treatment levels) and 84% inactivated by 20 min at 0.4% mg/L free available chlorine (municipal water treatment levels).

## ORGANISM INFORMATION

Taxonomy Information.

The genus *Clostridium* contains over 200 species of which *C. botulinum*, *C. difficile*, *C. perfringens*, and *C. tetani* are the species of human importance.

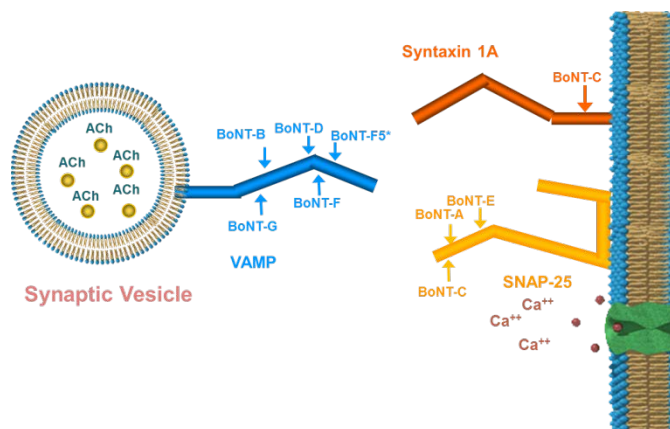
*C. botulinum* has been categorized into four physiological and genetic groups that have overlap with the toxins produced. The groupings are also supported by metabolic, structural, salt and acid resistance, and other differences (7).

Phenotypic groups of <i>Clostridium botulinum</i>				
Properties	Group I	Group II	Group III	Group IV
Toxin Types	A, B, F	B, E, F	C, D	G
Proteolysis	+	-	weak	-
Saccharolysis	-	+	-	-
Optimal growth temperature	35-40°C	18-25 °C	40 °C	37 °C
Disease host	human	human	animal	-
Toxin gene	chromosome	chromosome	bacteriophage	plasmid
Close relatives	<i>C. sporogenes</i> , <i>C. putrificum</i>	<i>C. butyricum</i> , <i>C. beijerinickii</i>	<i>C. hemolyticum</i> , <i>C. novyi</i> type A	<i>C. subterminale</i> , <i>C. hemolyticum</i>
Adapted from <b>Hatheway 1988</b>				

### Virulence Factors.

Although *C. botulinum* has a number of virulence factors, including capsules and bacteriocins (antimicrobial peptides or proteins), the toxins are by far the most significant and relevant. There are seven recognized antigenic types of *C. botulinum*: A, B, C, D, E, F, and G that affect humans and animals differently. Types A and B are most commonly encountered in foods associated with soil contamination and cause human disease. Types C and D are typically associated with animal mortalities (birds, cows, and others) and Type E in aquatic environments and contaminated fish or other seafood. Type F is rarely encountered but causes human disease. Each toxigenic *Clostridium* produces a polypeptide of 150 kD which is activated by proteases after bacterial lysis. The active toxin consists of a heavy chain (H, 100 kD) and a light chain (L, 50 kD). The heavy chain consists of an amino-terminal 50 kD domain (H<sub>N</sub>) and a carboxy-terminal 50 kD domain (H<sub>C</sub>).

The toxins are zinc metalloproteases (meaning they need zinc as a cofactor) that cleave and inactivate specific cellular proteins essential for the release of the neurotransmitter acetylcholine, the chemical that neurons release in order to activate muscles. BoNT-A, -C, and -E cleave SNAP (synaptosomal-associated protein)-25; BoNT-B, -D, -F, and -G cleave synaptobrevin 2 (also called VAMP 2). Of the serotypes, only 1, BoNT-C, cleaves >1 site on a specific protein. In addition to cleaving SNAP-25, BoNT-C also cleaves syntaxin (3, 5, 23).



### Epidemiology and Endemic Areas

*C. botulinum* is widely distributed in soils and in sediments of oceans and lakes worldwide and across the United States.

## LABORATORY DIAGNOSTICS

### Biosafety Information

Biosafety in Microbiological and Biomedical Laboratories (BMBL) Containment Recommendations (5th Edition):

Biosafety level (BSL)-2 practices, containment equipment, and facilities are recommended for activities that involve the organism or the toxin including the handling of potentially contaminated food. Solutions of sodium hypochlorite (0.1%) or sodium hydroxide (0.1N) readily inactivate the toxin and are recommended for decontamination of work surfaces and for spills. Autoclaving of contaminated materials also is appropriate.

BSL-3 practices, containment equipment, and facilities are required for activities with a high potential for aerosol or droplet production, and for those involving large quantities of the organism or of the toxin. Animal BSL-2 practices, containment equipment, and facilities are recommended for diagnostic studies and titration of toxin.

#### Diagnostic Information.

*Clostridium botulinum* is an anaerobic, gram positive, sporeforming rod, motile, 0.5-2.0 µm in width by 1.6-22.0 µm in length, with oval, subterminal spores.

#### Staining

Gram stain: They generally stain gram-positive but some *Clostridium* spp. show weak or negative Gram stain, especially in older cultures. Spores may be visible without special staining are subterminal, making the cell form a 'tennis racket' or 'drumstick' appearance.

#### Metabolic Information.

##### General.

Optimal temperature: Optimum temperature for growth and toxin production of proteolytic strains is close to 35°C; for non-proteolytic strains it is 26-28°C. Non-proteolytic types B, E, and F can produce toxin at refrigeration temperatures (3-4°C). Toxins of the non-proteolytics do not manifest maximum potential toxicity until they are activated with trypsin; toxins of the proteolytics generally occur in fully (or close to fully) activated form.

- (a) Upper temperature: When grown above 45°C, the bacteria become attenuated or avirulent due to loss of the capsule.
- (b) Lower temperature: 3-4°C (Group II, non-proteolytic strains).
- (c) Optimal pH: pH 7.0-7.4

General Culturing Information. *C. botulinum* grows well on most media incubated under anaerobic (without oxygen) conditions.

- (1) Chopped meat-glucose-starch (CMGS) medium.
- (2) Trypticase-peptone-glucose-yeast (TPGY) broth.
- (3) Egg yolk agar medium (lecithinase production-whitish-opaque surrounding colonies; lipase production-iridescent sheen on colonies).
- (4) Trypticase-peptone-glucose-yeast extract-trypsin medium.
- (5) Phenylethyl alcohol blood agar (PEA).
- (6) CDC anaerobe blood agar (beta hemolytic).
- (7) Columbia blood agar with yeast extract, vitamin K and hemin.
- (8) Cooked meat medium (CMM; for good toxin production).

Groups I-III form 2-8 mm diameter, translucent or semi-opaque with a rhizoid peripheral edge in 24-28 h. Some colonies will have a raised center with a yellowish color. Colonies on blood agar will have a single zone of beta hemolysis (as opposed to the double zone of hemolysis with *C. perfringens*). Group IV will form smaller and more translucent colonies, often spreading.

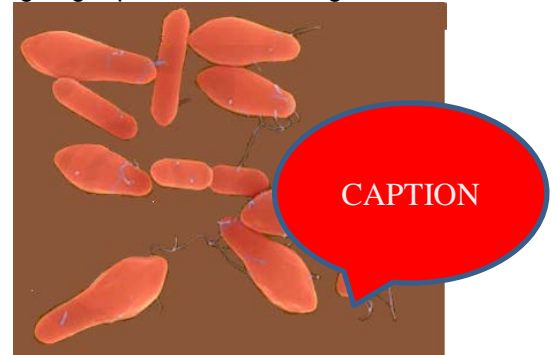
NOTE: Trypsin treatment: Toxins of non-proteolytic types, if present, may need trypsin activation to be detected.

#### Selective Culturing Information.

None identified.

#### Diagnostic Tests

Generally, laboratory confirmation is obtained by bioassay (mouse neutralization). Other helpful assays include enzyme-linked immunosorbent assay (ELISA) or electrochemiluminescence (ECL), or polymerase chain reaction (PCR) for bacterial DNA. Routinely, culture is rarely used due to time constraints and the necessity for anaerobic culture methods and media. Culture, however, can provide confirmation and contribute to attribution.



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If culture is used, presumptive identification of *C. botulinum* is based on cell physiology (anaerobic), morphology (gram-positive rods with oval, subterminal spores (in < 2 day old cultures), motile testing (motile), lecithinase (negative), lipase (positive), and indole (negative).

## Commercial Identification systems

Commercial test systems that are based on biochemical reactions have been developed for the identification of anaerobic bacteria but reports are contradictory on the ability of these tests to identify *Clostridium* spp. Commercial identification systems have been able to correctly identify 54% to 96% of the clostridial strains studied to the species level (30).

Fatty acid methyl ester (FAME) analysis: Simple gas-liquid chromatography of butyric and other short-chain fatty acid end-products has long been a presumptive indicator of *C. botulinum* (37). The commercial fatty acid methyl ester adaption also does a good job of discriminating the various *Clostridium* spp. when using the MOORE Anaerobe Broth Library; one of several libraries distributed by Microbial ID, Inc. (17). The MOORE library uses broth (PYG-Tween (PYG-T)) instead of solid media and is easier and more reproducible for *Clostridium* spp.

Biolog: While the GN2 system had a Dangerous Pathogens database for identification, *C. botulinum* requires the AN Database for Anaerobic bacteria. Little information on the use of Biolog for anaerobic bacteria is available but the system should be sufficient for at least genus identification.

Vitek. The Vitek 2, using the ANC ID Card for anaerobic and coryneform bacteria identification, will identify 89 taxa, representing 25 genera/groups (including 17 specific and 1 "Clostridium group"). Since *Cl. botulinum* is not specifically identified, the Vitek will return a variety of results including *Clostridium bifermentans*, *Clostridium difficile*, and *Clostridium sporogenes*, and none consistently.

Biolog. The Biolog anaerobic plate lists 361 taxa, including 60 different *Clostridia* spp. and specifically *Cl. botulinum*.

Commercial biochemical tests: Rapid ID 32 A, RapID ANA II and the API 20 A all have *C. botulinum* in their databases but none of them were highly specific for *C. botulinum* strains. Many were misidentified and *C. sporogenes* (non-toxigenic *C. botulinum* strains) were often confused. There were also differences between the assays between the proteolytic and non-proteolytic *C. botulinum* strains (31).

## Immunoassay Tests

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

## Direct agent immunoassays.

Serological testing. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

There are seven recognized antigenic types of *C. botulinum*: A, B, C, D, E, F, and G. Antigenic types of *C. botulinum* are identified by the complete neutralization of their toxins using the homologous antibody. Mixed toxin production by a single strain of *C. botulinum* may be more common than previously realized resulting in potential for inconclusive results in some testing scenarios.

NOTE: Food samples or anaerobic isolates need preparation for toxin production and are inoculated into TPGY (without trypsin) or CMM are incubated for 5 days at 26°C and 35°C, respectively, centrifuged at 7,000 X g and 4°C for 30 min, the supernatant is adjusted to pH 7.4-7.6.

Enzyme-linked immunoassays (ELISA): Standard ELISA have been devised and used to detect *C. botulinum*: A, B, C, D, E, F, and G toxins (10, 13, 14, 46, 49). DIG-ELISA, or digoxigenin-labeled antibody reagents are detected with anti-digoxigenin poly-HRP conjugate (35, 43, 45). In DIG-ELISA, wells of a microtiter plate are coated with goat type A, E, or F or rabbit type B antibodies in bicarbonate buffer as the capture for the toxin followed by the detection with the digoxigenin-labeled antibody. DIG-ELISA currently has found favor in the public health laboratories but consistent performance and reagent expiration are problematic.

Lateral flow immunochromatography: Hand-held assays (HHA), "Smart tickets" are commercially available and provide potentially useful. Current HHAs are typically limited to *C. botulinum* A, B, and

maybe E toxins. These are probably sufficient for most operational use but if diagnostic evaluation is negative, and that does not match operational intelligence, additional testing should be done (1, 44).

**Electrochemiluminescence (ECL):** Electrochemiluminescence as a detector system enhances the detection of many analytes. In the case of botulinum toxin B, ECL enhanced detection 2 to 4 times more sensitive than ELISA (18). In that study using only BotNT B complexes, the detection limit of the commercial colorimetric ELISA was 1.56 ng/ml versus 0.39–0.78 ng/ml in the M1R (M1M equivalent) ECL test. The M1M ECL assay used a paramagnetic bead-based ECL technology in which biotinylated serotype-specific antibodies were bound to streptavidin-coated paramagnetic beads to detect *C. botulinum* neurotoxins serotypes A, B, E, and F. ECL detection relies on the use of ruthenium chelate-labeled anti-serotype antibodies (41). The newer ECL format by MSD, the PR2, uses a well-based format with capture antibodies immobilized on a membrane in a spot array format. For Bot A, they claim a detection limit of 0.005–0.015 ng/ml of sample.

**Time-resolved fluorescence assays for *C. botulinum* A/B neurotoxin:** The assay format consists of a capture ELISA utilizing a biotinylated capture antibody, prebound to a streptavidin-coated 96-well plate and a lanthanide (europium, Eu3+)-labeled detector antibody. The bound Eu-labeled detector antibody produces a fluorescent signal upon the dissociation of the europium from the antibody, creating a micelle, thus amplifying the signal nearly one million-fold (39).

**Optical immunoassay (OIA):** OIA is based on detection of physical changes in the thickness of molecular thin film resulting from antibody binding on an optical silicon chip. The immunocomplex causes interference of a particular wavelength of reflected white light from gold to purple-blue (16).

**Large immuno-sorbent surface area (ALISSA):** BoNT/A ALISSA is a botulinum neurotoxin serotype A assay using 50 µm protein A beads and a fluorogenic peptide (SNAPTide) that fluoresces upon cleavage with the captured botulinum toxin on the beads (2).

**Liposome PCR assay:** An immunoassay that uses liposomes with encapsulated DNA reporters and ganglioside receptors embedded in the liposome bilayer as a detection reagent, have been described (36). The assay binds the toxin on a solid phase (microtiter plate) with a capture antibody and co-binds the liposomes. The liposomes are ruptured to release the reporters which are detected by real-time PCR. Assays have been reported to be “several orders of magnitude more sensitive than current detection methods” but have not been independently confirmed.

#### Nucleic Acid Detection Tests

**General PCR-based Assays:** PCR assays have been used for the detection of *C. botulinum* toxin genes in sample types. PCR-based assays detect genetic sequences of the organism, typically the toxin gene, not the toxin molecule itself. This is important to consider, since the organism may not be present in samples or the organism may be present and not involved in an intentional release of botulinum toxin (8, 9, 11, 12, 15, 29)

**Immuno-PCR:** Immuno PCR measures antigen-antibody reactions using a conjugated reporter DNA molecule followed by PCR amplification (6, 50).

#### Other Diagnostic Methods.

**Mouse bioassay:** The mouse bioassay is considered the “**gold-standard**” **functional assay** that detects biologically active toxin (13, 25). Simplistically, mice injected with the toxin develop respiratory failure and die. In confirmation, the toxin is neutralized with specific antibodies before testing and the mouse survives. The assay requires a three-part approach: toxin screening, toxin titer, and finally toxin neutralization using monovalent antitoxins. The process requires 2 to 7 days to complete.

- a. Signs of botulism in mice begin with ruffling of the fur followed in sequence by labored abdominal breathing and the characteristic ‘wasp-waist’ appearance. This is followed by weakness of the limbs and eventual total paralysis. Death is caused by respiratory failure. The time between the first sign of distress and death varies with the amount of toxin the mouse receives. Death without clinical signs is not adequate evidence that botulinum toxin was present in the material injected.
- b. Trypsinization. To 3.6 ml of culture, adjusted to pH 6.0–6.2, add 0.4 ml of 5% solution of trypsin. Incubate at 35–37°C for 1 h. Remove culture and let cool to room temperature before injecting mice. Trypsinized extract cannot be stored overnight.
- c. Toxicity screening. Dilute trypsinized and non-trypsinized broth cultures to 1:5, 1:10, and 1:100 in gel-phosphate diluent. (NOTE: Do not store trypsinized material overnight.) Inject mice i.p. with 0.5 ml of each dilution. Inject 2 mice per dilution, i.e., trypsinized and non-trypsinized (total 12 mice per subsample). Observe mice for botulism symptoms and record

condition of mice at frequent intervals for 48 h. If no deaths occur, no further tests are indicated. Deaths are presumptive evidence of toxin and should be confirmed.

- d. Confirmation with protected mice. Dilute new portion of non-trypsinized or trypsinized culture (whichever showed the highest titer) to 1:5, 1:10, and 1:100 in gel-phosphate diluent. (Do not store trypsinized material overnight.) Inject 6 mice i.p. with 0.5 ml of 1:5 saline dilution of type antiserum. These will be compared to 6 mice without this protection (controls). After 30 min, inject 0.5 ml of each dilution into 2 mice protected with antiserum and into 2 mice not so protected. Record their condition at intervals up to 48 h. If unprotected mice die and protected mice live, the presence of toxin is indicated. If all protected mice die, repeat confirmation with higher dilutions of toxic culture in type protected mice.
- e. NOTES:
  - (1) The first 24 h are the most important time regarding symptoms and death of mice: 98-99% of animals die within 24 h. Typical symptoms of botulism and death may occur within 4 to 6 h.
  - (2) Food and water may be given to the mice right away; it will not interfere with the test.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS): This method uses the protease activity of the toxins with target peptides specific for each toxin serotype and the subsequent detection of the product peptides detected by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (3). The Endopep-MS Assay is currently in deployment to public health laboratories as a confirmation method for detection of BoNT/A, /B, /E, and /F at levels below the standard mouse bioassay in clinical samples, such as serum and stool, along with culture supernatants and foods(4, 5, 24, 22, 40, 42).

Performance of <i>C. botulinum</i> toxin assays.				
Test method	Sample Type	Detection Limit (fg/mL)	Assay Time	Reference
Optical immunoassay (OIA)	BoNTs A, B, E, and F in buffer, water and food matrices	2,500,000-20,000,000	10-15 mins	Ganapathy 2008
Lateral-Flow immunoassay	BoNT types A, B, and E	10,000,000-20,000,000	15-20 mins	Sharma 2005
Mass spectrometry	BoNT complex types A-G in milk, serum, stool extract	320,000	4 h	Kalb 2005-2006 Boyer 2005 Barr 2005
Electrochemiluminescence (ECL)	BoNT A, B, E, and F in serum, urine, assay buffer, and selected food matrices	50,000-100,00	1 hr	Rivera 2006
	BoNT A	5,000-15,000	1.5 hr	Unpublished/tentative
DIG-ELISA	BoNT complex types A, B, E, and F in liquid and solid foods, serum	60,000	6-8 h	Sharma 2006
ELISA-HRP	BoNT type A in therapeutic preparations	9,000	4-6 h	Ekong 1995
Time-resolved fluorescence immunoassay (TRF)	BotNT A/B	4,000-20,000	2 h	Peruski 2002
Mouse assay	All sample types	5,000	~48 h	Kautter 1977; Smith 1988
Enzyme-amplified protein microarray immunoassay	BoNT type A in blood, plasma	1,400	10 min.	Varnum 2006
Immuno-PCR	BoNT types A, B, and E in carbonate buffer	50	4-6 h	Chao 2004 Wu 2001
ALISSA	BoNT type A in serum, milk, carrot juice, gelatin phosphate diluent	0.5	2-3 h	Bagramyan 2008
Liposome PCR assay	BoNT type A in deionized water	0.02	6 h	Mason 2006
Human lethal dose		0.1 – 70 ug		
Adapted from Bagramyan 2008 and Lindstrom 2006				

#### Characterization.

Characterization of *C. botulinum* strains can be done on cultured isolates, not directly on the toxins. Information on the *C. botulinum* strains can, in some cases, provide toxin information as well as defining the potential for forensic investigation of an event.

Ribotyping: An automated ribotyping system has been to study various strains of *C. botulinum*. Strains of *C. botulinum* from type A, proteolytic type B, non-proteolytic type B, and type E, have been



tested using the enzyme *EcoRI*. In one study, fifteen ribogroups were identified but in another study, type E was unable to be differentiated (20, 27, 47).

Variable number tandem repeat (VNTR): VNTR analysis of *C. botulinum* was shown to be capable of discriminating among type A and E strains (34, 33).

Pulsed-field gel electrophoresis (PFGE): A modified PFGE protocol (50  $\mu$ M thiourea) was judged to be the most useful method for typing epidemiologically related type E strains, based on its ability to type all strains reproducibly and with an adequate level of discrimination (19, 27, 28, 32, 38, 48)

Randomly amplified polymorphic DNA analysis (RAPD): RAPD analysis of *C. botulinum* type E strains was not consistently reproducible with primer OPJ-6 or OPJ-13, apparently discriminating between epidemiologically related strains (21, 27).

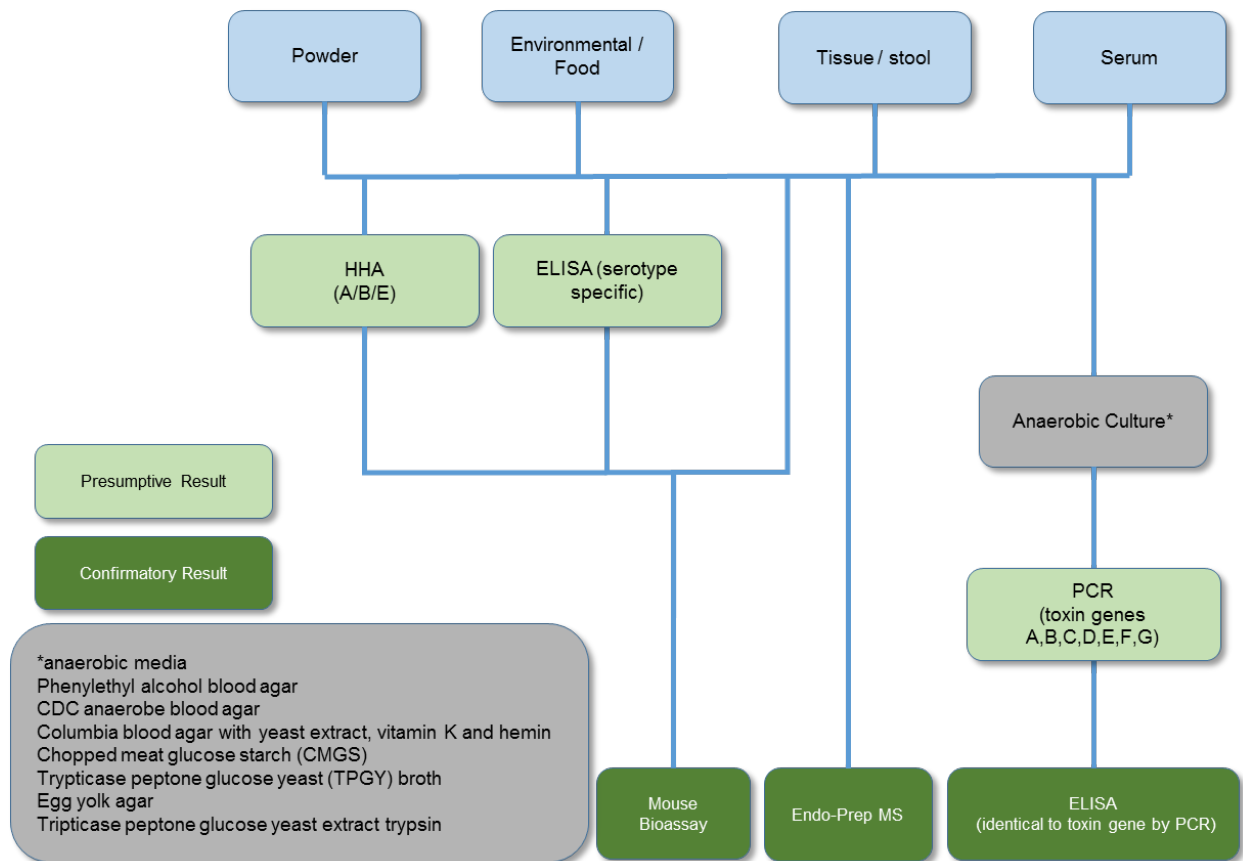
Amplified fragment length polymorphism (AFLP): AFLP analysis has been applied to characterize group I and group II *C. botulinum* strains. Using the enzyme combination *HindIII* and *HpyCH4IV*, with primers having one selective nucleotide apiece (*Hind-C* and *Hpy-A*) differentiated between *C. botulinum* groups I and II. Extensive diversity was observed among strains of *C. botulinum* type E, whereas group I had lower genetic differences in this study (26).

#### Confirmation

Presumptive identification of *C. botulinum*:

Lecithinase negative, lipase positive, indole negative.

Confirmation requires demonstration of toxin production.



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# Ricin

## OVERVIEW

Ricin is a potent protein cytotoxin derived from the beans of the castor plant (*Ricinus communis*). Castor beans are ubiquitous worldwide, and the toxin is easy to extract; therefore, ricin is widely available (2). When inhaled as a small particle aerosol, this toxin may produce pathologic changes within 8 hours and severe respiratory symptoms followed by acute hypoxic respiratory failure in 36-72 hours. When ingested, ricin causes severe gastrointestinal symptoms followed by vascular collapse and death; but large doses are necessary to obtain symptoms. The LD<sub>50</sub> of ricin by inhalation or injection is around 22 micrograms per kilogram (1.76 mg for an average adult). The oral LD<sub>50</sub> of ricin is far less toxic; around 20–30 milligrams per kilogram (~2 g for an average adult). While it has been claimed that if the beans are chewed or broken, oral ingestion of 5-20 castor beans could prove fatal to an adult and it is said 1 bean can kill a child, this information is viewed with much skepticism.

Ricin's significance as a potential biowarfare toxin relates in part to its wide availability. Worldwide, one million tons of castor beans are processed annually in the production of castor oil; the waste mash from this process is 3-5 percent ricin by weight. The toxin is also quite stable and extremely toxic by several routes of exposure, including the respiratory route. In 1962, a US patent was issued for "Toxic ricin for warfare" (3). Ricin was apparently used in the assassination of Bulgarian exile Georgi Markov in London in 1978 (10). Markov was attacked with a specially engineered weapon disguised as an umbrella, which implanted a ricin-containing pellet into his body. This technique was used in at least six other assassination attempts in the late 1970s and early 1980s. Ricin has a high terrorist potential due to its ready availability, relative ease of extraction, and notoriety in the press.

## TOXIN CHARACTERISTICS

Ricin is classified as a type 2 ribosome inactivating protein (RIP) and made up of a toxin component and a hemagglutinin component (as opposed to a type 1 RIP that are single chain proteins). The toxin and hemagglutinin form a dimer with a molecular mass of about 66,000 daltons. The toxins are made up of two polypeptide chains, an A chain and a B chain, which are joined by a disulfide bond. The A chain has the endonuclease activity and at extremely low concentrations will inhibit DNA replication and protein synthesis. The B chain is what binds to cell-surface receptors and transfers toxin the cell (20).

Ricin, *Ricinus communis* agglutinin (RCA), and other terms exist because some castor bean varieties contain different agglutinins (RCL I and RCL II) as well as potentially being composed of at least three different isoforms (ricin D, RCL-III, etc.) with similar molecular structures with different isoelectric points (pI; molecule with no net electrical charge).

*Ricinus communis* agglutinin (RCA) occurs in two forms designated RCA-60 (RCAII, Ricin D or RCL III) and RCA-120 (RCAI /RCA-1 or RCL (I + II)) according to their molecular weight of approximately 60,000 and 120,000 respectively. RCA-120 is a tetrameric protein but has reduced toxicity. RCA-60 (most appropriately called ricin is a dimeric AB toxin composed of an A-chain (RTA, ricin toxin A chain) with a disulphide-linkage to a B-chain (RTB, ricin toxin B chain). RTA is an N glycosidase, responsible for ricin cytotoxicity, that depurinates adenine residue 4324 of the 28S rRNA, thus inactivating protein synthesis. Since its endocytotic properties are very limited, RTA alone shows a very low level of cytotoxicity. RTB is a galactose specific lectin containing three galactose-binding sites and alone shows no cytotoxicity. Mutation or deletion of any one of these sites reduces the ability of ricin to penetrate into the cell.

Ricin A Chain (RTA) is an N-glycoside hydrolase composed of 267 amino acids (~28-32 kDa). It has three structural domains with approximately 50% of the polypeptide arranged into alpha-helices and beta-sheets. The three domains form a pronounced cleft that is the active site of RTA.

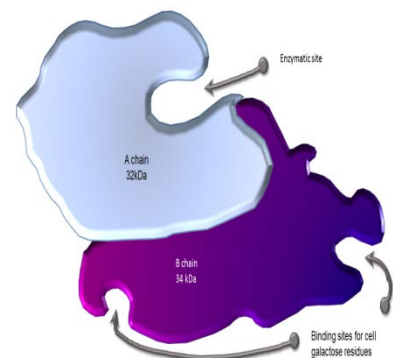


Figure 53. Ricin A chain B chain structure

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Ricin B Chain (RTB) is a lectin composed of 262 amino acids (~32-34 kDa) that is able to bind terminal galactose residues on cell surfaces. RTB form a bilobal, barbell-like structure lacking alpha-helices or beta-sheets where individual lobes contain three subdomains. At least one of these three subdomains in each homologous lobe possesses a sugar-binding pocket that gives RTB its functional character.

**The various proteins that comprise ricin and agglutinins will cause problems for several detection methods. The active ricin molecule of interest is RCA-60, composed of RTA and RTB. Neither RTA nor RTB are toxic when separated. RCA-120 is also inactive as a tetrameric protein.**

It can be disseminated as an aerosol, injected, or used to contaminate food or water; although the latter is relatively unfeasible due to the oral LD<sub>50</sub> being so high. Ricin is stable under ambient conditions, but is detoxified by heat (80°C for 10 minutes or 50°C for about an hour at pH 7.8) and chlorine (>99.4 percent inactivation by 100 mg/L free chlorine in 20 minutes). Low chlorine concentrations, such as 10 mg/L free chlorine, as well as iodine at up to 16 mg/L, have no effect on ricin. Ricin's toxicity is marginal when comparing its LD<sub>50</sub> to other toxins, such as botulinum and SEB (incapacitating dose).

An enemy would need to produce it in large quantities to cover a significant area on the battlefield, limiting its large-scale use. Large quantities of ricin can be produced relatively easily and inexpensively by low-level technology. Ricin can be prepared in a liquid suspension, in crystalline form, or it can be lyophilized to make a dry powder.

*Ricinus communis* is grown as an ornamental in gardens, sometimes as a houseplant, and also grows as a weed. It is a perennial in the south. This can present problems for laboratory diagnostics, especially, nucleic acid tests where trace contamination can cause a false-positive assay.

Other plants with ribosomal inhibiting proteins	
Plant Family	Plant species
Adoxaceae	<i>Sambucus nigra</i> , <i>S. sieboldiana</i> (Elderberry trees)
Amaryllidaceae	<i>Agave americana</i> (century plant, maguey, or American aloe)
Caryophyllaceae	<i>Agrostemma githago</i> (corncockle), <i>Dianthus caryophyllus</i> (Clove Pink), <i>Saponaria officina</i> (soapwort, saporin)
Celastaceae	<i>Evonimus europaeus</i>
Chenopodiaceae	<i>Beta vulgaris</i> , <i>Chenopodium album</i> , <i>Chenopodium amaranticolor</i> , <i>Spinacia oleracea</i>
Cucurbitaceae	<i>Bryonia alba</i> , <i>Bryonia dioica</i> , <i>Cirullus colocynthis</i> , <i>Cucurbita pepo</i> , <i>Ecballium elaterium</i> , <i>Momordaa balsamina</i> , <i>Momordica charantia</i>
Euphorbiaceae	<i>Acalipha indica</i> (Indian acalypha, Indian nettle), <i>Aleurites fordii</i> , <i>Croton bonplandianum</i> , <i>Croton tiglium</i> , <i>Euphorbia lathyris</i> , <i>Euphorbia pilulijera</i> , <i>Gelonium multiflorum</i> , <i>Revea brasiliensis</i> , <i>Rura crepitans</i> , <i>Jatropha curcas</i> , <i>Jatropha gossypifolia</i> , <i>Jatropha multifida</i> , <i>Jatropha podagrica</i> (coral plant, physic nut, Guatemala rhubarb), <i>Mallotus philippinensis</i> , <i>Manihot palmata</i> , <i>Manihot utilissima</i> , <i>Putranjiba roxburghii</i> , <i>Ricinus communis</i> , <i>Trewia nudiflora</i>
Fabaceae	<i>Wisteria floribunda</i> (Japanese wisteria)
Gramineae	<i>Avena sativa</i> (common oat), <i>Hordeum vulgare</i> (barely), <i>Secale cereal</i> (rye), <i>Triticum aegilops</i> , <i>Triticum aestivum</i> , <i>Triticum dicoccoides</i> , <i>Triticum dicoccum</i> (wheat)
Leguminoseae	<i>Abrus precatorius</i> (jequirity bean; crabs eye; rosary pea), <i>Crotalaria juncea</i> (hemp) <i>Vida cracca</i> (Vetch)
Lillieaeae	<i>Asparagus officinalis</i>
Loranthaceae	<i>Phoradendron serotinum</i> (Oak (or Eastern) mistletoe), <i>Viscum album</i> (European mistletoe)
Passifloraceae	<i>Adenia digitata</i> , <i>Adenia volkensii</i>
Phytolaccaceae	<i>Phytolacca americanav</i> (pokeweed)
Rhamnaceae	<i>Rhamnus cathartica</i> (buckthorn)
Sapindaceae	<i>Koelreuteria paniculata</i> (goldenrain)
Solanaceae	<i>Datura stramonium</i> (Jimson weed)



Adapted from Jimenez 1985 (8) and Peumans 2001 (15)

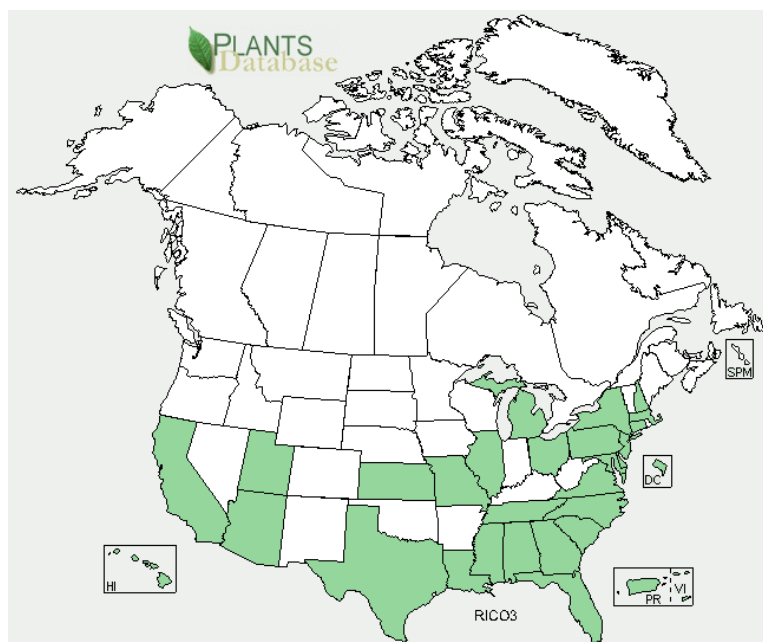


Figure 54. Distribution of *Ricinus communis* in North America (green states).

## LABORATORY DIAGNOSTICS

### Biosafety Information.

Biosafety in Microbiological and Biomedical Laboratories (BMBL) Containment Recommendations (5th Edition): Biosafety level (BSL)-2 practices, containment equipment and facilities are recommended, especially a laboratory coat, gloves, and protective mask, when handling ricin toxin or potentially contaminated materials. Ricin is a relatively non-specific cytotoxin that should be handled in the laboratory as a non-volatile toxic chemical. A biosafety cabinet (Class II, Type B1 or B2) or a chemical fume hood equipped with an exhaust HEPA filter and charcoal filter are indicated for activities with a high potential for aerosol, such as powder samples, and the use of large quantities of toxin. Laboratory coat, gloves, and full face respirator should be worn if there is a potential for creating a toxin aerosol.

### Diagnostic Information.

Ricin is a ribosome-inactivating protein (RIP); culture and routine microbiological procedures do not apply.

Ricin is an extremely immunogenic toxin. While serum, urine, and respiratory secretions could be submitted for antigen detection (immunoassay), the clinical diagnostic utility of assays for ricin in those specimens is not known. It has been said that ricin can be detected in plasma within 1 day, but detection in urine requires 3 days (11). Acute and convalescent sera provide the best retrospective diagnosis. Specific immunoassay tests of serum and respiratory secretions, or immunohistochemical stains of tissue may be used where available to confirm the diagnosis. The best samples for ricin testing are liquid suspensions or powders of the actual threat material. PCR can detect castor bean DNA in most ricin preparations.

### Immunoassay Tests

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

Direct agent immunoassays.

**Serological testing.** The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers

between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

Enzyme-linked immunosorbent assay (ELISA): Reported limits of detection (LOD) for ELISA range from 0.1 to 10 ng/mL (1, 6, 12, 16, 18).

Time-resolved fluorescence immunoassay (TRF): Anti-ricin detects ricin in environmental samples.

Lateral flow immunochromatography: Hand-held assays (HHA), "Smart tickets", or lateral flow assays are commercially available and provide potentially useful for environmental samples (19).

Method	Detection limits	Note	Reference
LD <sub>50</sub>	1.76 mg for an average adult = 352 ng/ml blood		
ELISA	0.1-10 ng/mL		Cook 2006 Griffiths 1986
TRF	1-10 ng/ml*		
ECL	0.1-2 ng/ml*	M1M	unpublished*
	0.004-0.01 ng/ml	MSD PR2	unpublished*
HPLC-ESI-MS	0.083 ng/mL (urine)	ricinine 1:5 to ricin; sublethal dose may be reflected by a ricinine concentration below 0.08 ng/mL.	Johnson 2005
HHA	3.6* - (25*) 50 ng/ml		Shyu 2002
Bead-based	0.002-1 ng/ml	MagPix	unpublished*
*unpublished but valid sourced; currently subject to specific lot production			

#### Nucleic Acid Detection Tests:

Polymerase chain reaction (PCR): PCR is a test used to locate and make copies of parts of the DNA contained in the castor bean plant. The PCR assay can specifically look for the DNA of the gene that produces the ricin protein or may target other genes in the plant genome. Several PCRs have been developed but most have not been published (17). One published method is a duplex real-time PCR assay that simultaneously detects ricin and abrin DNA (5).

An immuno-polymerase chain reaction (IPCR) assay for the detection of RIPs that combines the specificity of immunological analysis with the exponential amplification of PCR has also been developed (7, 14).

#### Other diagnostic methods:

A cell-free translation (CFT) assay for determining ricin biological activity has been developed and validated. The assay was specific for determining ricin biological activity in food-based matrixes and discriminated ricin from other ribosome-inactivating proteins (13).

HPLC-ESI-MS: A method for measuring ricinine (a marker of ricin exposure) in urine has been developed but is not widely available and the usefulness of this assay is questionable. The method limit of quantification was 0.083 ng/mL. Ricinine was stable in human urine when heated at 90°C for 1 h, and during storage at 25°C and 5°C for 3 weeks. Sample collection should occur within 48 h (2, 4, 9)

#### Characterization

Efforts to genetically characterize *Ricinus communis* from various parts of the world is underway, but the value seems questionable, even for forensics, with the wide dispersion of the plant world wide.

#### Confirmation



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# Staphylococcal Enterotoxin B

## OVERVIEW

*Staphylococcus aureus* produces a number of exotoxins (at least 17), including eight distinct toxins that have been implicated in human intoxication; SEA, SEB, SEC1, SEC2, SEC3, SED, SEE, TSST-1. Such toxins are referred to as exotoxins as they are excreted from the organism, and as they normally exert their effects on the intestines, they are called enterotoxins. SEB is one of the pyrogenic toxins that commonly cause food poisoning in humans after the toxin is produced in improperly handled foodstuffs and is subsequently ingested. SEB has a very broad spectrum of biological activity. This toxin causes a markedly different clinical syndrome when inhaled than it characteristically produces when ingested. Significant morbidity is produced in individuals who are exposed to SEB by either portal of entry to the body.

SEA and SEB are the most common source of outbreaks of food poisoning. Often these outbreaks occur in a setting such as a church picnic or other community event, due to common-source exposure in which contaminated food is consumed. In biowarfare, SEB would be the most likely weaponized version. Although an aerosolized SEB toxin weapon would not likely produce significant mortality, it could render 80 percent or more of exposed personnel clinically ill and unable to perform their mission for 1-2 weeks. The demand on the medical and logistical systems could be overwhelming.

## TOXIN CHARACTERISTICS

Staphylococcal enterotoxins are proteins of 23-29 kilodaltons molecular mass (SEB is 28,494 daltons). They are extracellular products of coagulase-positive staphylococci. Up to 50% of clinical isolates of *S. aureus* produce exotoxins. These toxins are moderately stable; SEB is inactivated after a few minutes at 100°C. Ingestion of food containing at least 0.05-1 µg of toxin can cause a mild form of food poisoning with a rapid onset of symptoms (2-4 h), including nausea and vomiting with abdominal cramps, occasionally followed by diarrhea. Recovery is usually completed within 48 h and deaths are very rare. If inhaled, however, inhalation LD<sub>50</sub> in humans may be as low as 20 ng/kg SEB (19).

## ORGANISM INFORMATION

### Taxonomy Information

More than 70 species of *Staphylococci* are described but only *Staphylococcus aureus* and *Staphylococcus epidermidis* are significant in their interactions with humans, with *S. aureus* being the primary toxin producer.

### Virulence Factors.

1. Surface proteins that promote colonization of host tissues
2. Invasins that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase)
3. Surface factors that inhibit phagocytic engulfment (capsule, Protein A)
4. Biochemical properties that enhance their survival in phagocytes (carotenoids, catalase production)
5. Immunological disguises (Protein A, coagulase)
6. Membrane-damaging toxins that lyse eucaryotic cell membranes (hemolysins, leukotoxin, leukocidin)
7. Exotoxins that damage host tissues or otherwise provoke symptoms of disease (SEA-G, TSST, ET)
8. Inherent and acquired resistance to antimicrobial agents.

### Epidemiology and Endemic Areas.

World-wide with little significance to intoxication.

## LABORATORY DIAGNOSTICS

### Biosafety Information

Biosafety in Microbiological and Biomedical Laboratories (BMBL) Containment  
Recommendations (5th Edition):

Biosafety level (BSL)-2 practices and containment equipment and facilities should be used when handling SE or potentially contaminated material. Because SE is highly active by the oral or ocular exposure route, the use of a laboratory coat, gloves and safety glasses is mandatory when handling toxin or toxin-contaminated solutions. Frequent and careful hand-washing and laboratory decontamination should be strictly enforced when working with SE. Depending upon a risk assessment of the laboratory operation, the use of a disposable face mask may be required to avoid accidental ingestion.

BSL-3 facilities, equipment, and practices are indicated for activities with a high potential for aerosol or droplet production and those involving the use of large quantities of SE.

### Diagnostic Information

*Staphylococcus aureus* is a gram-positive coccal bacterium that is a member of the Firmicutes, and is frequently found in the nose, respiratory tract, and on the skin. It is often positive for catalase and nitrate reduction. Although *S. aureus* is not always pathogenic, it is a common cause of skin infections such as abscesses, respiratory infections such as sinusitis, and food poisoning. Pathogenic strains often promote infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. The emergence of antibiotic-resistant forms of *S. aureus* such as MRSA is a worldwide problem in clinical medicine.

*Staphylococcus* was first identified in 1880 in Aberdeen, Scotland, by the surgeon Sir Alexander Ogston in pus from a surgical abscess in a knee joint.[1] This name was later appended to *Staphylococcus aureus* by Friedrich Julius Rosenbach, who was credited by the official system of nomenclature at the time. An estimated 20% of the human population are long-term carriers of *S. aureus*[2] which can be found as part of the normal skin flora and in the nostrils.[2][3] *S. aureus* is the most common species of *Staphylococcus* to cause Staph infections and is a successful pathogen due to a combination of nasal carriage and bacterial immunoevasive strategies.[2][3] *S. aureus* can cause a range of illnesses, from minor skin infections, such as pimples,[4] impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is still one of the five most common causes of hospital-acquired infections and is often the cause of postsurgical wound infections. Each year, around 500,000 patients in United States' hospitals contract a staphylococcal infection like *S. aureus*.[5]

### KEY BIOCHEMICAL REACTIONS

Catalase-positive.

Oxidase-negative.

Urease-variable.

Ornithine-decarboxylase-negative.

Nitrate is often reduced to nitrate.

Commonly halotolerant.

**Staph are xxx..x.x.x.x.x.x.x..xxxxx**

Staining.

Gram stain: Gram-positive bacteria, cocci, in clusters, 0.5-1.0 µm in diameter.

### Metabolic Information:

General: *Staphylococcus* spp. are facultative anaerobes and grow well on most media. They are chemoorganotrophic, having both a fermentative and respiratory type metabolism.

Optimal temperature: 35-37°C.

(a) Upper temperature: 45°C.

(b) Lower temperature: 15°C.

Optimal pH: pH range is 4.5 - 9.3, with an optimum of 7.0 - 7.5.

(2) Note: NaCl tolerance up to 15%.

### General Culturing Information.

## DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

After overnight incubation at 35-37°C colonies are smooth, entire, slightly raised, large cream white/grey to light golden yellow, 1-3 mm in diameter, after 24 h, and 8 mm after 3 d. On sheep blood agar the colonies are strongly beta hemolytic.

- i) Sheep (horse) blood agar (SBA).
- ii) Nutrient agar (NA).
- iii) Trypticase (tryptic) soy agar (TSA).
- iv) Tryptone yeast extract agar.

Baird-Parker medium: Colonies are circular, smooth, convex, moist, 2-3 mm in diameter, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle.

Toluidine blue-DNA agar: DNase activity of *S. aureus* is indicated by a pink to red zones surrounding growth.

#### Selective Culturing Information.

Mannitol salt agar: A selective medium with 7–9% NaCl that allows *S. aureus* to grow, producing yellow-colored colonies as a result of mannitol fermentation and subsequent drop in the medium's pH.

#### Diagnostic Tests

*Staphylococcus* spp. is gram-positive, catalase positive, non-motile, non-sporeforming cocci. The most important toxigenic species is *S. aureus*, although other species, such as *S. intermedius* may also produce toxins. Routine culture of suspect samples (clinical or environmental) does not directly indicate the presence of toxins but isolation of toxigenic *Staphylococci* could lead to attribution.

Laboratory confirmation of intoxication includes antigen detection (ELISA, ECL) on environmental and clinical samples, and gene amplification (like PCR to detect staphylococcal genes) on environmental samples.

SEB may not be detectable in the serum by the time symptoms occur; regardless, a serum specimen should be drawn as early as possible after exposure. Data from rabbit studies clearly show that the presence of SEB in the serum is transient; however, accumulation in the urine can be detected for several hours post-exposure. Urine samples, therefore, should also be obtained and tested for SEB.

Respiratory secretions and nasal swabs may demonstrate the toxin early (within 24 h of exposure).

Because most patients develop a significant antibody response to the toxin, acute and convalescent sera should be drawn for retrospective diagnosis but anti-staphylococcal antibodies are common in most people.

Coagulase test: Coagulase plasma (EDTA rabbit plasma) is inoculated with a small portion of an 18-24 hr culture and incubate at 35-37°C for up to 6 h (37). Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus*.

#### Ancillary tests:

- (1) Catalase test: Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is used to test growth for the catalase test; observe production of gas bubbles.
- (2) Anaerobic utilization of glucose and mannitol: Inoculate tube of carbohydrate fermentation medium containing glucose or mannitol (0.5%). Cover surface of agar with layer of sterile paraffin oil at least 25 mm thick. Incubate 5 days at 35-37°C. Acid is produced anaerobically if indicator changes to yellow throughout tube. Anaerobic utilization of glucose is positive for *S. aureus*. Anaerobic utilization of mannitol is usually positive but some strains are negative.
- (3) Lysostaphin sensitivity: Emulsify a portion of an 18-24hr culture in phosphate-saline buffer and add 0.1 ml lysostaphin (25 µg lysostaphin/ml). Incubate both tubes at 35-37°C for not more than 2 h. Clearing of turbidity in 2 h is considered positive. *S. aureus* is generally positive.
- (4) Thermostable nuclease production: This test is claimed to be as specific as the coagulase test but less subjective, because it involves a color change from blue to bright pink. It is not a substitute for the coagulase test but rather is a supportive test, particularly for 2+ coagulase reactions. Microslides are prepared by spreading 3 ml toluidine blue-deoxyribonucleic acid agar on the surface of each microscope slide, 2 mm diameter wells are cut in agar and removed. 0.01 ml of heated sample (15 min in boiling water bath) of broth cultures is added to well and incubated in a moist chamber for 4 h at 35-37°C. Development of bright pink halo extending at least 1 mm from well indicates a positive reaction.



Some typical characteristics of staphylococci and the micrococci, which may be helpful in their identification, are shown.

Typical characteristics of <i>S. aureus</i> , <i>S. epidermidis</i> , and micrococci <sup>(a)</sup>			
Characteristic	<i>S. aureus</i>	<i>S. epidermidis</i>	Micrococci
Catalase activity	+	+	+
Coagulase production	+	-	-
Thermonuclease production	+	-	-
Lysostaphin sensitivity	+	+	-
Anaerobic utilization of glucose	+	+	-
mannitol	+	-	+
Bacitracin (0.04 U, Taxo A disk)	R	S	S
DNase Test Agar	+	-	-
<sup>a</sup> +, Most (90% or more) strains are positive; -, most (90% or more) strains are negative.			

Commercial Identification systems: All commercial identification systems all include the ability to identify *S. aureus*. Commercial systems typically show agreement to conventional microbiological methods from around 70 to 87%, suggesting that some care must be exercised in *Staphylococcus* spp. identification methods. The fatty acid methyl ester (FAME) analysis system (9, 20, 24), the Vitek (6, 10, 12, 25), and the Biolog : (22, 24) have *S. aureus* in their databases.

#### Immunoassay Tests

Immunoassays can be used to detect either the organisms directly or the antibody response to the organism by a host (serology).

#### Direct agent immunoassays.

Serological testing. The use of paired serum samples, taken during the acute and convalescent phases of the disease, or at 1-to 2-week intervals, is preferable for serological tests. Demonstration of a rise in titers between the acute and convalescent samples, or a single sample with a 'significant' titer is needed to interpret serological assays.

Antibody titers after exposure are of little value as many individuals contain antibodies to these toxins and the antibodies are cross-reactive, however, a rise from acute to convalescent serum samples should be considered as presumptive evidence of intoxication.

Enzyme-linked immunosorbent assay (ELISA): Several various ELISA have been developed to detect the most common staphylococcal enterotoxins (2, 11, 27, 34) and some assays are commercially available. Two commercially available assays that are popular and cited in the FDA Bacteriological Analytical Manual (<http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm073674.htm>) specifically identify staphylococcal enterotoxins A, B, C, D and E, providing results within hours. The commercial VIDAS SET2 is used as the primary screening method for suspect food samples and culture isolates and the commercial TECRA<sup>TM</sup> polyvalent system should be used as a confirmatory test and the TECRA<sup>TM</sup> monovalent kit as a valuable tool to identify the specific staphylococcal enterotoxin serotype(s). False positive reactions are problematic due to inherent protein A binding. Protein A is a protein found in the cell wall of *S. aureus* that strongly binds immunoglobulins, predominately IgGs causing the potential for false-positive results if the antibodies are IgG based. For the commercial assays, special care and attention to Protein A problems have been addressed and eliminated.

Lateral flow immunochromatography: Hand-held assays (HHA), "Smart tickets", or lateral flow assays are commercially available and provide potentially useful. 1 ng/ml of SEB can be detected in less than 5 min using a lateral flow assay.

Electrochemiluminescence (ECL): Detection of SEB in the linear ranges (0.1–100 ng/ml) has been demonstrated with ECL (17). MSD PR2, the more recent ECL platform, has claimed a sensitivity of 0.0005-0.001 ng/ml for their system (unpublished).

Reversed passive latex agglutination (RPLA): A commercial RPLA-based test for toxin detection is available that detects SEA to SED toxins (3, 14). Variations to this assay exist.

Western immunoblotting: A Western immunoblotting assay was reported for SEA (28).

Time-resolved fluorometry (TRF): *Staphylococcus aureus* enterotoxin B (SEB) was converted to a TRF assays and tested in different matrices such as serum, urine, dirt, and sewage (26).

### Nucleic Acid Detection Tests

General PCR-based Assays: There are several types of staph enterotoxins (A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, and R) and the corresponding genes (sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sel, sem, sen, seo, sep, seq, ser and seu) have been described (4, 5, 13, 18, 21, 31-33, 36). SEA, SEC, and SEE are all phage-borne, while SED is plasmid-encoded. Insufficient data are available to clearly define the nature of SEB and TSST-1 genetic elements. In addition to PCR identification of the toxin genes directly, the thermostable nuclease (*nuc*) and 60-kD heat-shock protein (*hsp60*) genes have been described (5, 7).

### Other diagnostic methods

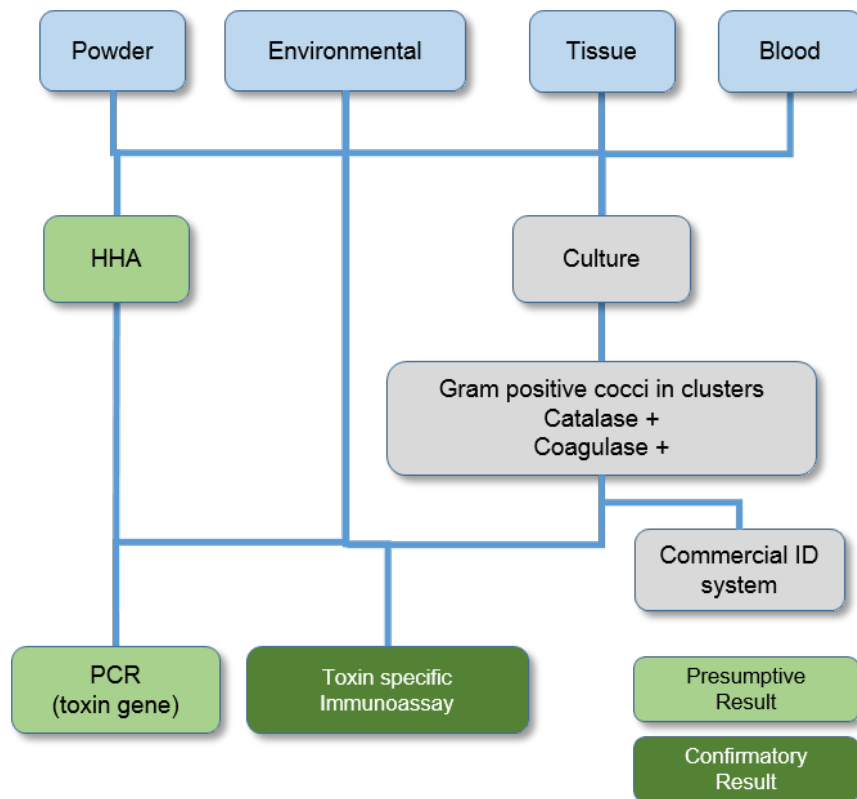
Cell based assays: A T-cell proliferation assay that measures SEA activity in food has been described. In the assay human or rat lymphocytes proliferated in response to SEA as low as 1 pg/ml to 1 ng/ml (29). In another assay, IFN-gamma production in mouse splenocytes or primary naive CD4+ T-cells were used to demonstrate biologically active SEA (30).

### Characterization

*Staphylococcus aureus* has been well characterized by several methods (phage typing, pulsed-field gel electrophoresis (PFGE), *spa* typing, and multilocus sequence typing (1, 8, 15, 16, 23, 35, 38, 39) but the usefulness for toxin differentiation use has not been evaluated.

### Confirmation

Demonstration of specific toxin is required.



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## Appendix A: Glossary of Terms

Ab - Abbreviation for antibody.

Adenosine triphosphate (adenosine 5'-triphosphate)(Abbreviation: ATP) - A nucleotide of fundamental importance as the major carrier of chemical energy in all living organisms. It is also required for RNA synthesis since it is a direct precursor molecule. ATP consists of adenosine with three phosphate groups, linked together linearly. The phosphates are attached to adenosine through the 5'-hydroxyl of its ribose (sugar) portion. Upon hydrolysis, these bonds yield either one molecule of adenosine 5'-diphosphate (ADP) and the inorganic phosphate ion, or one molecule of adenosine 5'-monophosphate (AMP) and pyrophosphate; in both cases releasing energy that is used to power biological processes. ATP is regenerated by the phosphorylation of AMP and ADP.

Aerobe - A micro-organism that grows in the presence of oxygen. Opposite: anaerobe.

Aerobic - Active in the presence of free oxygen, e.g. aerobic bacteria that can live in the presence of oxygen.

Aerobic respiration - A type of respiration in which foodstuffs are completely oxidized to carbon dioxide and water, with the release of chemical energy, in a process requiring atmospheric oxygen.

Affinity chromatography - A method for purifying specific components in a solution by exploiting their specific binding to known molecule(s). The mixed solution is passed through a column containing a solid medium to which the binding molecule is covalently attached. See: immunoaffinity chromatography; metal affinity chromatography; pseudo-affinity chromatography.

Aflatoxins - A group of toxic compounds, produced by *Aspergillus flavus* that bind to DNA and prevent replication and transcription. Aflatoxins can cause acute liver damage and cancer. A health hazard in certain stored foods or feed.

AFLP - Abbreviation for amplified fragment length polymorphism

Ag - Abbreviation for antigen.

Agar - A polysaccharide gelifying agent used in nutrient media preparations and obtained from Rhodophyta (red algae). Both the type of agar and its concentration can affect the growth and appearance of cultured explants.

Agarose - The main functional constituent of agar.

Agarose gel electrophoresis - A method to separate DNA and RNA molecules on the basis of their size, in which samples are subjected to an electric field applied to a gel made with agarose

AHF - Argentine hemorrhagic fever, a viral hemorrhagic fever

Aleukia - Absence or extremely decreased number of leukocytes in the circulating blood.

Amplicon The product of a DNA amplification reaction. See: polymerase chain reaction.

Amplification - 1. Creation of many copies of a segment of DNA by the polymerase chain reaction. 2. Treatment (e.g. use of chloramphenicol) designed to increase the proportion of plasmid DNA relative to that of bacterial (host) DNA. 3. Evolutionary expansion in copy number of a repetitive DNA sequence through a process of repeated duplication.

Amplified fragment length polymorphism (Abbreviation: AFLP) - A type of DNA marker, generated by the PCR amplification of restriction endonuclease treated DNA. A small proportion of all restriction fragments is amplified in any one reaction, so that AFLP profiles can be analyzed by gel electrophoresis. This has the important characteristic that many markers can be generated with relatively little effort.

Amplify - To increase the number of copies of a DNA sequence, either in vivo by inserting into a cloning vector that replicates within a host cell, or in vitro by polymerase chain reaction.

Anaerobe - An organism that can grow in the absence of oxygen. Opposite: aerobe.

Anaerobic - An environment or condition in which molecular oxygen is not available for chemical, physical or biological processes.

Anaerobic respiration Respiration in which foodstuffs are partially oxidized, with the release of chemical energy, in a process not involving atmospheric oxygen. A notable example is in alcoholic fermentation, where sugar is metabolized into ethanol.

Antibiotic - A class of natural and synthetic compounds that inhibit the growth of, or kill some microorganisms. Antibiotics are widely used medicinally to control bacterial pathogens, but resistance in bacteria to particular antibiotics is often rapidly acquired through mutation.

Antibody (Abbreviation: Ab) - An immunological protein produced by the lymphocytes in response to contact with an antigen. Each antibody recognizes just one antigenic determinant of one antigen and acts by specifically binding to it, thus rendering it harmless. Those from the IgG antibody class are found in the bloodstream and used in immunoassay. Synonym: immunoglobulin. See: monoclonal antibody, polyclonal antibody.

Antibody binding site - The part of an antibody that binds to the antigenic determinant. See: complementarity-determining regions. Synonym: paratope.

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**Antibody class** - The class to which an antibody belongs, depending on the type of heavy chain present. In mammals, there are five classes of antibodies: IgA, IgD, IgE, IgG, and IgM.

**Antibody structure** - Describes the molecular architecture of an antibody, which consists of two identical "light" chains and two identical "heavy" chains and has two antigen-binding sites. Each chain consists of a constant region which is the same between antibodies of the same class and sub-class, and a variable region that is antibody-specific.

**Antibody-mediated immune response** - The synthesis of antibodies by B cells in response to an encounter of the cells of the immune system with a foreign antigen. Synonym: humoral immune response.

**Antigen (Abbreviation: Ag)** - A macromolecule (usually a protein foreign to the organism), which elicits an immune response on first exposure to the immune system by stimulating the production of antibodies specific to its various antigenic determinants. During subsequent exposures, the antigen is bound and inactivated by these antibodies. Synonym: immunogen.

**Antimicrobial agent** - Any chemical or biological agent that inhibits the growth and/or survival of microorganisms.

**Antitoxin** - An antibody formed in response to and capable of neutralizing a biological poison; an animal serum containing antitoxins.

**Apoptosis** The process of programmed cell death, which occurs naturally as a part of normal development, maintenance and renewal of tissue. Differs from necrosis, in which cell death is caused by external factors (stress or toxin).

**AP-PCR** - See: arbitrarily primed polymerase chain reaction.

**Aptamer** - A polynucleotide molecule that binds to a specific molecule, often a protein.

**Arbitrarily primed polymerase chain reaction (Abbreviation: AP-PCR)** - An application of the polymerase chain reaction to generate DNA fingerprints. The technique uses arbitrary primers to amplify anonymous stretches of DNA. See: DNA amplification fingerprinting, random amplified polymorphic DNA.

**Ascites** - Abnormal accumulation of fluid in the peritoneal cavity, occurring naturally as a complication of cirrhosis of the liver, among other conditions. In the context of monoclonal antibody production, hybridoma cells are injected into mice to induce their proliferation in the resulting ascites. This method has been largely superseded by in vitro culture of hybridomas.

**Assay** - 1. To test or evaluate. 2. The procedure for measuring the quantity of a given substance in a sample (chemically or by other means).

**Auxotroph** - A mutant cell or micro-organism lacking one metabolic pathway present in the parental strain, and that consequently will not multiply on a minimal medium, but requires for growth the addition of a specific compound, such as an amino acid or a vitamin.

**Bacillus** - A rod-shaped bacterium.

**Bacteriocin** - are proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strain(s).

**Bacteriophage (Abbreviation: phage)** - A virus that infects bacteria. Altered forms are used as cloning vectors.

**Bacteriostat** - A substance that inhibits or slows down growth and reproduction of bacteria.

**Bacterium (pl.: bacteria)** - Unicellular prokaryotic organisms, without a distinct nucleus. Major distinctive groups are defined by Gram staining. Also classified on the basis of oxygen requirement (aerobic vs. anaerobic) and shape (spherical = coccus; rodlike = bacillus; spiral = spirillum; comma-shaped = vibrio; corkscrew-shaped = spirochaete; filamentous).

**Base** - One of the components of nucleosides, nucleotides and nucleic acids. Four different bases are found in naturally occurring DNA - the purines A (adenine) and G (guanine); and the pyrimidines C (cytosine) and T (thymine, the common name for 5-methyluracil). In RNA, T is replaced by U (uracil). See: base pair.

**Base analogue** - A non-natural purine or pyrimidine base that differs slightly in structure from the normal bases, but can be incorporated into nucleic acids. They are often mutagenic.

**Base pair (Abbreviation: bp)** - The two separate strands of a nucleic acid double helix are held together by specific hydrogen bonding between a purine and a pyrimidine, one from each strand. The base A pairs with T in DNA (with U in RNA); while G pairs with C in both DNA and RNA. The length of a nucleic acid molecule is often given in terms of the number of base pairs it contains.

**Bio-informatics** - The use and organization of information of biological interest. In particular, concerned with organizing bio-molecular databases (particularly DNA sequences), utilizing computers for analyzing this information, and integrating information from disparate biological sources.

**Biological containment** - Restricting the movement of organisms from the laboratory. Can take two forms: making the organism unable to survive in the outside environment, or making the outside environment inhospitable to the organism. For micro-organisms, the favored approach is to engineer organisms to require a supply of a specific nutrient that is usually available only in the laboratory. For higher organisms (plants and animals), it is more possible to ensure that the outside environment is unsuited to growth, spread and reproduction.



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**Biosafety** - Referring to the avoidance of risk to human health and safety, and to the conservation of the environment, as a result of the use for research and commerce of infectious or genetically modified organisms.

**Biological safety cabinet** – (biosafety cabinet, BSC) is an enclosed, ventilated laboratory workspace for safely working with materials contaminated with (or potentially contaminated with) dangerous microbial organisms. The purpose of a BSC is to serve as a means to protect the laboratory worker and the surrounding environment from pathogens. Exhaust air is filtered as it exits the biosafety cabinet, removing harmful microbial organisms (bacteria, fungi, or viruses). There are three classes of BSC with classes 2 and 3 being the most common and most protective.

**Blood agar** - A mixture of blood and nutrient agar used for the cultivation of many medically important microorganisms.

**Blot** - As a verb, to transfer DNA, RNA or protein to an immobilizing matrix. As a noun, the immobilizing matrix carrying DNA, RNA or protein. The various types of blot are named according to the probe and/or the probed molecules: Southern blot (DNA/DNA), northern blot (DNA/mRNA), western blot (antibody/protein), southwestern blot (DNA/protein). Only "Southern" is written with an initial capital, as it is named after Ed Southern, the inventor of the technique.

**bp** - Abbreviation for base pair.

**BSC** – see Biological safety cabinet.

**Bubo** - Inflammatory swelling of one or more lymph nodes, usually in the groin; the confluent mass of nodes usually suppurates and drains pus.

**Bulla**, gen. and pl. **bullae** - A large blister appearing as a circumscribed area of separation of the epidermis from the subepidermal structure (subepidermal bulla) or as a circumscribed area of separation of epidermal cells (intraepidermal bulla) caused by the presence of serum, or occasionally by an injected substance.

**Butyrous** - Denoting a tissue or bacterial growth of butter like consistency.

**Capillary electrophoresis** - A form of electrophoresis used widely in current large-scale DNA sequencing facilities, where the sample is passed through a long, very-narrow-bore tube containing a re-usable matrix.

**Capsid** - The protein coat of a virus. The capsid often determines the shape of the virus. Synonym: coat protein.

**Capsule** - Carbohydrate coverings that have antigenic specificity, present on some types of bacteria and other microorganisms. The capsule is usually composed of polysaccharides, polypeptides, or polysaccharide-protein complexes. These materials are arranged in a compact manner around the cell surface.

**Carbuncle** - Deep-seated pyogenic infection of the skin and subcutaneous tissues, usually arising in several contiguous hair follicles, with formation of connecting sinuses; often preceded or accompanied by fever, malaise, and prostration.

**Catabolic pathway** - A pathway by which an organic molecule is degraded in order to release energy for growth and other cellular processes.

**Catabolite repression** - Glucose-mediated reduction in the rates of transcription of genes that encode enzymes involved in catabolic pathways (e.g. the lac operon).

**Catalase** - A metalloenzyme, present in both plants and animals that catalyzes the decomposition of hydrogen peroxide to water and oxygen. This activity is important in the detoxification of reactive oxygen generated as part of the response to stress.

**CCHF** - Congo-Crimean hemorrhagic fever, a viral hemorrhagic fever

**Centrifugation** - Separating molecules by size or density using centrifugal forces generated by a spinning rotor. G-forces of several hundred thousand times gravity are generated in ultracentrifugation.

**Chemically-defined medium** - When all of the chemical components of a culture medium are fully known and defined.

**Chromosome** - In eukaryotic cells, chromosomes are the nuclear bodies containing most of the genes largely responsible for the differentiation and activity of the cell. Chromosomes are most easily studied in their contracted state, which occurs around the metaphase of mitosis or meiosis; they contain most of the cell's DNA in the form of chromatin. Each eukaryotic species has a characteristic number of chromosomes. Bacterial and viral cells contain only one chromosome, which consists of a single or double strand of DNA or, in some viruses, RNA, without histones.

**Coat protein** - See: capsid.

**Coccobacillus** - A short, thick bacterial rod of the shape of an oval or slightly elongated coccus.

**Codon** - One of the groups of three consecutive nucleotides in mRNA, which represent the unit of genetic coding by specifying a particular amino acid during the synthesis of polypeptides in a cell. Each codon is recognized by a tRNA carrying a specific amino acid, which is incorporated into a polypeptide chain during protein synthesis. In DNA, any informative triplet of bases, including both coding and control sequences. See: genetic code, start codon, stop codon.

**Colony** - 1. A group of genetically identical cells or individuals derived from a single progenitor. 2. A group of interdependent cells or organisms.

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Complementary DNA (Abbreviation: cDNA) - A DNA strand synthesized in vitro from a mature RNA template using reverse transcriptase. DNA polymerase is then used to create a double-stranded molecule. Differs from genomic DNA by the absence of introns. Synonym: copy DNA.

Contaminant - 1. An undesired chemical present in a compound or mixture of compounds. 2. Any micro-organism accidentally introduced into a culture or culture medium. The contaminant may compete with the desired cells and consequently inhibit their growth, or totally replace them.

cry proteins - A class of crystalline proteins produced by strains of *Bacillus thuringiensis*, and engineered into crop plants to give resistance against insect pests. These proteins are toxic to certain categories of insects (e.g. corn borers, corn rootworms, mosquitoes, black flies, armyworms, tobacco hornworms, some types of beetles, etc.), but are harmless to mammals and most beneficial insects. Synonym: delta endotoxins.

Cryoprotectant - Compound preventing cell damage during successive freezing and thawing processes. Cryoprotectants are agents with high water solubility and low toxicity. Two types commonly used: permeating (glycerol and DMSO) and non-permeating (sugars, dextran, ethylene glycol, polyvinylpyrrolidone and hydroxyethyl starch).

Cytopathic effect (CPE) - Refers to structural changes in the cells that are caused by viruses. Viruses can cause lysis of the host cell or changes to the cell morphology. Examples of CPE include rounding of the cell, fusion with adjacent cells to form syncytia (multinucleated cell that can result from cell fusions of uninuclear cells), and the appearance of nuclear or cytoplasmic inclusion bodies.

CPEs and other changes in cell morphology are only a few of the many effects by cytotoxic viruses. When a cytotoxic virus infects a permissive cell, the viruses kill the host cell through changes in cell morphology, in cell physiology, and the biosynthetic events that follow. These changes are necessary for efficient virus replication but at the expense of the host cell.

Denatured DNA - Double-stranded DNA that has been converted to single strands by breaking the hydrogen bonds linking complementary nucleotide pairs. Often reversible. Usually achieved by heating.

Diagnostic procedure - A test or assay used to determine the presence of a specific substance, organism or nucleic acid sequence alteration, etc.

Diffusion - The spontaneous movement of molecules from a region of higher concentration to a region of lower concentration.

Digest - To treat DNA molecules with one or more restriction endonucleases in order to cleave them into smaller fragments.

Dimer - 1. A molecule formed by the covalent combination of two monomers, generally accompanied by elimination of water. 2. The reversible association of two similar (or nearly similar) molecules. The active form of many enzymes is as a dimer between two non-active monomeric subunits.

Disinfection - Attempted elimination by chemical means of internal microorganisms (particularly pathogens) from a culture or sample; rarely attained.

DNA - Abbreviation for deoxyribonucleic acid, former spelling desoxyribonucleic acid. A long chain polymer of deoxyribonucleotides. DNA constitutes the genetic material of most known organisms and organelles, and usually is in the form of a double helix, although some viral genomes consist of a single strand of DNA, and others of a single- or a double-stranded RNA. See: base pair, genetic code.

DNA amplification - Many-fold multiplication of a particular DNA sequence either in vivo in a plasmid, phage or other vector; or in vitro using, most commonly, the polymerase chain reaction.

DNA diagnostics - The use of DNA polymorphisms to detect the presence of a specific sequence, which could indicate the presence of a contaminant, a pathogen, or a particular allele at a target gene. Most commonly utilizes the polymerase chain reaction.

DNA fingerprint - A description of the genotype of an individual from the pattern of DNA fragments obtained from DNA fingerprinting. Synonym: DNA profile.

DNA fingerprinting - The derivation of unique patterns of DNA fragments obtained using a number of marker techniques; historically these were RFLPs, but latterly they are generally polymerase chain reaction based. Synonym: genetic fingerprinting.

DNA helicase - An enzyme that catalyses the unwinding of the complementary strands of a DNA double helix. Synonym: gyrase.

DNA hybridization - The annealing of two single-stranded DNA molecules, possibly of different origin, to form a partial or complete double helix. The degree of hybridization varies with the extent of complementarity between the two molecules, and this is exploited to test for the presence of a specific nucleotide sequence in a DNA sample.

DNA ligase - An enzyme that catalyses a reaction to link two separate DNA molecules via the formation of a phosphodiester bond between the 3'-hydroxyl end of one and the 5'-phosphate of the other. Its natural role lies in DNA repair and replication. An essential tool in recombinant DNA technology, as it enables the incorporation of foreign DNA into vectors.

DNA polymerase - See: polymerase.

DNA polymorphism - The existence of two or more alternative alleles at a DNA-based marker locus.

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DNA sequencing - Procedures for determining the nucleotide sequence of a DNA fragment. Two common methods available: 1. The Maxam Gilbert technique, which uses chemicals to cleave DNA into fragments at specific bases; or, most commonly, 2. the Sanger technique (also called the di-deoxy or chain-terminating method) which uses DNA polymerase to make new DNA chains, in the presence of di-deoxynucleotides (chain terminators) to stop the chain randomly as it grows. In both cases, the DNA fragments are separated according to length by polyacrylamide gel electrophoresis, enabling the sequence to be read directly from the gel. The procedure has become increasingly automated and large-scale in recent years.

DNase - Abbreviation for deoxyribonuclease. Any enzyme that catalyses the cleavage of DNA phosphodiester bonds. DNase I is a digestive endonuclease secreted by the pancreas that degrades DNA into shorter fragments. Many other endonucleases and exonucleases are involved in DNA repair and replication. Synonym: DNAase. See: restriction endonuclease.

ECL - See below for Electrochemiluminescence.

Edema - An accumulation of an excessive amount of watery fluid in cells, tissues, or serous cavities.

EDTA - See: ethylenediamine tetraacetic acid.

Electrochemiluminescence - A method used to identify microorganisms. It is a relatively new technique for this purpose and is similar in operation to ELISA, FA and sandwich antibody assays. A capture antibody bound to a magnetic bead captures the target microorganism. Another antibody labeled with a ruthenium tris-bipyridyl compound ( $\text{Ru}(\text{bpy})_3^{2+}$ ) is introduced. A magnet is used to pull the beads to an electrode which is used to excite the ruthenium compound which then emits light. The light is detected revealing the presences of the target organism. The method is easily automated and is generally faster than either ELISA or FA.

Electron microscope (Abbreviation: EM) - A microscope that uses an electron beam focused by magnetic 'lenses'. See: scanning electron microscope.

Electrophoresis - A ubiquitous molecular biology technique, with many variants, used to resolve complex mixtures of macromolecules into their components. Its principle is to subject samples to an electric field applied across a porous matrix. Molecules will migrate under these conditions at a rate dependent on their net electric charge and/or their molecular weight. See: agarose gel electrophoresis, polyacrylamide gel electrophoresis, denaturing gradient gel electrophoresis, capillary electrophoresis, sodium dodecyl sulphate polyacrylamide gel electrophoresis, thermal gel gradient electrophoresis pulsed-field gel electrophoresis, and iso-electric focusing gel.

Electroporation - The induction of transient pores in bacterial cells or protoplasts by the application of a pulse of electricity. These pores allow the entry of exogenous DNA into the cell. Widely used for the transformation of bacteria.

ELISA - Abbreviation for enzyme-linked immunosorbent assay. An immunoassay, i.e. an antibody-based technique for the diagnosis of the presence and quantity of specific molecules in a mixed sample. It combines the specificity of an immunoglobulin with the detectability of an enzyme-generated colored product. In one form, the primary antibody (specific to the test protein) is adsorbed onto a solid substrate, and a known amount of the sample is added; all the antigen in the sample is bound by the antibody. A second antibody (conjugated with an enzyme) specific for a second site on the test protein is added; and the enzyme generates a color change in the presence of a substrate reagent.

ELISA - See below for Enzyme Linked Immunosorbent Assay.

Encephalitis, pl. encephalitides - Inflammation of the brain.

Enterotoxin - A cytotoxin specific for the cells of the intestinal mucosa.

Enzyme immunoassay - A range of immunoassay techniques employing enzymes, which includes ELISA.

Enzyme Linked Immunosorbent Assay - A method used to detect microorganisms such as bacteria or viruses or toxins. It works by chemically linking an enzyme to an antibody that recognizes and adheres to the desired microorganism. Any unbound antibody-enzyme complex is removed and chemical which is converted by the enzyme into a fluorescent compound is applied and allowed to react. The fluorescence is then detected to reveal the presence or absence of the microorganism.

Enzyme-linked immunosorbent assay - See: ELISA.

Epistasis - Interaction between genes at different loci, e.g. one gene suppresses the effect of another gene that is situated at a different locus. Dominance is associated with members of allelic pairs, whereas epistasis describes an interaction among products of non-alleles.

Epistaxis - Profuse bleeding from the nose.

Epizootic - 1. Denoting a temporal pattern of disease occurrence in an animal population in which the disease occurs with a frequency clearly in excess of the expected frequency in that population during a given time interval. 2. An outbreak (epidemic) of disease in an animal population; often with the implication that it may also affect human populations.

Erythema - Redness of the skin due to capillary dilatation.

Ethidium bromide - A fluorescent dye which can intercalate between base pairs of double-stranded DNA, and hence is much used to stain DNA in gels. The dye fluoresces when exposed to UV light. It is known to be a strong mutagen and is also possibly both a carcinogen and a teratogen.

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Ethyl alcohol - See: ethanol.

Ethylenediamine tetraacetic acid (Abbreviation: EDTA) - A chelating compound. Used to keep nutrients, such as iron, bound in a soluble form that leaves them still available to the plant cells in vitro. Also a potent inhibitor of DNase activity and therefore used as an additive for long-term storage of dissolved DNA.

Eukaryote - One of the two major evolutionary clades, characterized by having the nucleus enclosed by a membrane, and possessing chromosomes that undergo mitosis and meiosis. Eukaryotic organisms include animals, plants, fungi and some algae.

Excitation wavelength - The specific wavelength of light required to stimulate a fluorescent molecule, such as a labeled probe, to emit light at the (lower) emission wavelength.

Excrete - To transport material out of a cell or organism.

Exon - A segment of a eukaryotic gene that is transcribed as part of the primary transcript and is retained, after processing, with other exons to form a functional mRNA molecule. Many eukaryotic genes are composed of a mosaic of exons and introns.

Exonuclease - An enzyme that digests DNA or RNA, beginning at the end of a strand. It therefore requires a free end in order to begin the degradation. 5'-exonucleases require a free 5' end and degrade the molecule in the 5' > 3' direction. 3'-exonucleases require a free 3' end and degrade in the opposite direction.

FA - See below for fluorescent antibody.

Febrile - Denoting or relating to fever.

Fermentation - The anaerobic breakdown of complex organic substances, especially carbohydrates, by microorganisms, yielding energy. Often misused to describe large-scale aerobic cell culture in specialized vessels (fermenters, bioreactors) for secondary product synthesis.

Fermentation substrates - Materials used as food for growing microorganisms. The fermentation substrates and the trace materials needed, together with chemicals added to make the fermentation easier, form the culture medium.

Fingerprinting - See: DNA fingerprinting.

Fluorescence immunoassay (Abbreviation: FIA) - An immunoassay based on the use of fluorescence-labeled antibody.

Fluorescence in situ hybridization (Abbreviation: FISH) - Hybridization of cloned, fluorescently labeled DNA or RNA, to intact biological materials, notably chromosome spreads and thin tissue sections. The technique allows the visualization of the physical location of nucleic acid sequences homologous to the probe, and is used for the placement of genes on chromosomes and for the spatial and temporal pattern of gene expression of specific mRNA molecules.

Fluorescence Resonance Energy Transfer (FRET) - The excitation of a donor fluorescent dye is transferred to a receptor dye, leading to the fluorescence of the acceptor dye instead of the donor dye. The transfer is possible only if the two dyes are in close proximity. Applications include receptor-ligand interactions and real-time PCR assays

Fluorescent antibody - A method used in microbiology to detect microorganisms usually bacteria. An antibody with an attached fluorescent molecule is applied to a slide containing the bacteria and washed to remove unbound antibody. Under UV light the bacteria to which antibodies are bound will fluoresce revealing their presence.

Freeze-dry - The removal of water as vapor from frozen material under vacuum. Used to measure water content and to preserve samples, particularly vegetative cells. Unlike oven-drying, bound water remains associated with the specimen. Synonym: lyophilize.

Fungus (pl.: fungi) - Multinucleate single-celled or multicellular heterotrophic microorganisms, including yeasts, moulds, and mushrooms. They live as parasites, symbionts, or saprophytes. Lacking any vascular tissues (unlike plants), their cell walls are made of chitin or other non-cellulose compounds.

Generalized vaccinia - Secondary lesions of the skin following vaccination which may occur in subjects with previously healthy skin but are more common in the case of traumatized skin, especially in the case of eczema (eczema vaccinatum). In the latter instance, generalized vaccinia may result from mere contact with a vaccinated person. Secondary vaccinal lesions may also occur following transfer of virus from the vaccination to another site by means of the fingers (autoinnoculation).

Genome - 1. The entire complement of genetic material (genes plus non-coding sequences) present in each cell of an organism, virus or organelle. 2. The complete set of chromosomes (hence of genes) inherited as a unit from one parent.

Genomics - The research strategy that uses molecular characterization and cloning of whole genomes to understand the structure, function and evolution of genes and to answer fundamental biological questions. See: bio-informatics, functional genomics and proteomics.

Genotype - 1. The genetic constitution of an organism. 2. The allelic constitution at a particular locus, e.g. Aa or aa. 3. The sum effect of all loci that contribute to the expression of a trait.

Genus (pl.: genera) - A group of closely related species, whose perceived relationship is typically based on physical resemblance, now often supplemented with DNA sequence data.

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Glanders - A chronic debilitating disease of horses and other equids, as well as some members of the cat family, caused by *Burkholderia mallei*; it is transmissible to humans. It attacks the mucous membranes of the nostrils of the horse, producing an increased and vitiated secretion and discharge of mucus, and enlargement and induration of the glands of the lower jaw.

Good laboratory practice (Abbreviation: GLP) - Written codes of practice designed to reduce to a minimum the chance of procedural or instrument problems which could adversely affect a research project or other laboratory work. Typically applied to work with laboratory animals.

Good manufacturing practice (Abbreviation: GMP) - Codes of practice designed to reduce to a minimum the chance of procedural or instrument/manufacturing plant problems which could adversely affect a manufactured product.

Gram staining - A technique to distinguish between two major bacterial groups, based on whether or not their cell wall retains the Gram stain. Gram-positive bacteria are stained dark purple, while gram-negative bacteria are stained red to pink. Stain retention is determined by the structure of the cell wall.

Green fluorescent protein (Abbreviation: GFP) - A protein derived from a species of jelly fish, that fluoresces when exposed to ultra violet light. Its encoding gene has been isolated and is replacing GUS as a reporter gene in plant transgenesis, since it can be assayed non-destructively in real time.

Growth phase - Each of the characteristic periods in the growth curve of a bacterial culture, as indicated by the shape of a graph of viable cell number versus time, namely: lag phase; logarithmic phase; stationary phase; death phase.

Guarnieri bodies - Intracytoplasmic acidophilic inclusion bodies observed in epithelial cells in variola (smallpox) and vaccinia infections, and which include aggregations of Paschen body's or virus particles.

Gyrase - See: DNA helicase

Hemagglutination - The agglutination of red blood cells; may be immune as a result of specific antibody either for red blood cell antigens per se or other antigens which coat the red blood cells, or may be nonimmune as in hemagglutination caused by viruses or other microbes.

Hemagglutinin - A substance, antibody or other, that causes hemagglutination.

Hematemesis - Vomiting of blood.

Hemolysis - Alteration, dissolution, or destruction of red blood cells in such a manner that hemoglobin is liberated into the medium in which the cells are suspended, e.g., by specific complement-fixing antibodies, toxins, various chemical agents, tonicity, alteration of temperature.

Hemolytic Uremic Syndrome - Hemolytic anemia and thrombocytopenia occurring with acute renal failure.

HEPA (high-efficiency particulate arrestance) - a type of air filter that typically removes (from the air that passes through) 99.97% of particles that have a size of 0.3  $\mu\text{m}$  (removes microbial level organisms).

Heterotroph - Organism non capable of self-nourishment utilizing carbon dioxide or carbonates as the sole source of carbon and obtaining energy from radiant energy or from the oxidation of inorganic elements, or compounds such as iron, sulphur, hydrogen, ammonium and nitrites. Opposite: autotroph.

HFRS - Hemorrhagic Fever with Renal Syndrome. A viral hemorrhagic fever syndrome caused by viruses of the genus Hantavirus, Bunyaviridae family, with renal impairment as the primary organ manifestation.

Homology - 1. The degree of identity between individuals, or characters. 2. The degree of identity of sequence (nucleotide or amino acid) between a number of DNA or polypeptide molecules.

Hybridization - 1. The process of forming a hybrid by cross pollination of plants or by mating animals of different types. 2. The production of offspring of genetically different parents, normally from sexual reproduction, but also asexually by the fusion of protoplasts or by transformation. 3. The pairing of two DNA strands, often from different sources, by hydrogen bonding between complementary nucleotides.

Immunoaffinity chromatography - A purification technique in which an antibody is bound to a matrix and is used to isolate a protein from a complex mixture. See: affinity chromatography.

Immunoassay - A detection system for a particular molecule, which exploits the specific binding of an antibody raised against it. For measurement, the antibody can incorporate a radioactive or fluorescent label, or be linked to an enzyme which catalyses an easily monitored reaction such as a change in color (see: ELISA). Synonym: immunodiagnostics.

in silico - In a computer file. In the present context, the use of data bases of DNA and protein sequence to help answer biological questions. This is growing area of biology as the amount of genomics and proteomics data continues to grow. See: bio-informatics.

in situ - In the natural place or in the original place. 1. Experimental treatments performed on cells or tissue rather than on extracts from them. 2. Assays or manipulations performed with intact tissues.

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**in situ hybridization** - The visualization of in vivo location of macromolecules (particularly polynucleotides and polypeptides) by the histological staining of tissue sections or cytological preparations via labeled probes/antibodies.

**in vitro** - Outside the organism, or in an artificial environment. Applied for example to cells, tissues or organs cultured in glass or plastic containers.

**in vivo** - The natural conditions in which organisms reside. Refers to biological processes that take place within a living organism or cell under normal conditions

**Infectious agent** - Synonym of pathogen.

**Inoculation** - Introduction into the body of the causative organism of a disease.

**Insertion element** - Generic term for DNA sequences found in bacteria capable of genome insertion. Postulated to be responsible for site-specific phage and plasmid integration. Synonym: insertion sequence.

**Intercalating agent** - A chemical capable of inserting between adjacent base pairs in a double-stranded nucleic acid. A prominent example is ethidium bromide.

**Internal transcribed spacer (Abbreviation: ITS)** - Non-coding regions separating the individual components of the ribosomal DNA units. These regions show much more sequence polymorphism than the genic regions themselves, and therefore, like the intergenic spacers, are useful a source of genetic markers for the ribosomal DNA locus.

**Intron** - A segment of the primary transcript of a eukaryotic gene, removed (before the mature mRNA is translated) in a process known as intron splicing. Some eukaryotic genes contain a large number of introns, which make up the bulk of the DNA sequence of the gene. Introns are also found in genes whose RNA transcripts are not translated, namely eukaryotic rRNA and tRNA genes. In these cases the intron sequence does not appear in the functional RNA molecule. Synonym: intervening sequence.

**Inverted repeat** - Two sequences of nucleotides occurring in one strand, where, relative to the first sequence, the second has complementary bases but in the inverted order. Under appropriate conditions this allows formation of a hairpin loop in the single strand.

**IS element** - Abbreviation for insertion sequence element. A short (800-1400 nucleotide pairs) DNA sequence found in bacteria that is capable of transposing to a new genomic location; DNA sequences contained within an IS element can be transposed along with the IS itself.

**Kilobase (Abbreviation: kb)** - A length of single-stranded nucleic acid composed of 1000 bases. One kilobase of single-stranded DNA has a mass of about 330 kiloDalton (exact mass depends on base composition).

**Kilobase pairs (Abbreviation: kbp)** - A length of double-stranded DNA composed of 1000 base pairs.

**Label** - A compound or atom that is attached to, or incorporated into, another molecule in order to allow detection of the latter's presence. Commonly, labels exploit radioactivity, fluorescence or antigenicity. Synonym: tag.

**Labeling** - The process of attaching or inserting a label into a molecule. Most often in the context of nucleic acids or proteins.

**LCR** - Abbreviation for ligase chain reaction.

**LD<sub>50</sub>** - Abbreviation for lethal dose 50%. The amount of a substance required to kill 50% of the test population. The higher the LD<sub>50</sub>, the lower the toxicity of the agent in that specific test. In toxicology, the LD<sub>50</sub> of a particular substance is a measure of how much constitutes a lethal dose. In toxicological studies of substances, one test is to administer varying doses of the substance to populations of test animals; that dose administered which kills half the test population is referred to as the LD<sub>50</sub>.

**Leukocyte** - White blood cell, up to 0.02 mm in diameter, of which there are normally 4-11 million per millilitre of human blood. There are several kinds; all involved in the body's defense mechanisms. Granulocytes have granules in their cytoplasm; monocytes ingest and feed on bacteria and other microorganisms that cause infection; lymphocytes include the B cells that are involved with the production of antibodies.

**Leukopenia** - The antithesis of leukocytosis; any situation in which the total number of leukocytes in the circulating blood is less than normal, the lower limit of which is generally regarded as 4000-5000 per cu mm.

**Ligase chain reaction (Abbreviation: LCR)** - A technique for the detection and amplification of target DNA sequences. Two oligonucleotides are synthesized which between them are complementary to the entire target sequence, one to the 5'-side and one to the 3'-side. If the target sequence is present in the DNA sample under examination, the oligonucleotides will bind to it with their ends abutting in the centre, and a heat-stable ligase will join them into a complete polynucleotide. No ligation occurs if the target sequence is absent or if the match between synthetic oligonucleotides and target sequence is imperfect in the region where they abut. At a high temperature, the new polynucleotide dissociates from the original DNA template and upon cooling, it and the original DNA serve as templates for a second cycle of hybridization, ligation and thermal dissociation. At each cycle there is a doubling of the number of new complete polynucleotides.

**Ligase** - See: DNA ligase.

**Ligate, ligation** - The joining of two linear double-stranded DNA fragments by the formation of phosphodiester bonds.

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**Lipase** - A class of enzymes which break down lipids into their component fatty acids and glycerol. Lipases used in biotechnology are generally digestive, with a role in the break-down of fats in food into their components, so that these can be used to make other materials.

**Lipopolysaccharide (Abbreviation: LPS)** - A compound containing lipid bound to a polysaccharide; often a component of microbial cells walls.

**Logarithmic phase** - The growth phase in cell culture, during which cell number doubles every 20-30 min. Synonym: exponential phase.

**LPS** - Abbreviation for lipopolysaccharide.

**LTR** - Abbreviation for long terminal repeat.

**Lymphadenopathy** - Any disease process affecting a lymph node or lymph nodes.

**Lymphopenia** - A reduction, relative or absolute, in the number of lymphocytes in the circulating blood.

**Lyophilize** - See: freeze-drying.

**Lysis** - The destruction or breakage of cells either by viruses or by chemical or physical treatment.

**Lysogen** - A bacterial cell whose chromosome contains integrated bacteriophage DNA.

**Lysogenic** - Bacteria or bacteriophages undergoing lysogeny.

**Lysogenic bacterium** - Bacterium harboring temperate (non-virulent, lysogenic) bacteriophages.

**Lysogeny** - A condition in which a bacteriophage genome (pro-phage) survives within a host bacterium, either as part of the host chromosome or as part of an extrachromosomal element, and does not initiate lysis.

**Lysosome** - A membrane-bound sac within the cytoplasm of animal cells that contains enzymes responsible for the digestion of material in food vacuoles, the dissolution of foreign particles entering the cell and, on the death of the cell, the breaking down of all cell structures. The digestive system of the cell.

**Lysozyme** - A naturally occurring enzyme extracted from egg white protein and other animal and plant sources, which attacks the cell wall of gram-positive bacteria leading to cell lysis and death.

**Lytic** - A phase of the virus life cycle during which the virus replicates within the host cell, releasing a new generation of viruses when the infected cell undergoes lysis.

**Lytic cycle** - The steps in viral production that lead to cell lysis.

**Macula, pl. maculae** - 1. A small spot, perceptibly different in color from the surrounding tissue. 2. A small, discolored patch or spot on the skin, neither elevated above nor depressed below the skin's surface.

**Media** See: culture medium; medium.

**Mediastinitis** - Inflammation of the cellular tissue of the mediastinum.

**Mediastinum** - The median partition of the thoracic cavity, covered by the mediastinal pleura and containing all the thoracic viscera and structures except the lungs.

**Medium (pl.: media)** - 1. In plant tissue culture, a term for the liquid or solid formulation upon which plant cells, tissues or organs develop. See: culture medium. 2. In general terms, a substrate for plant growth, such as nutrient solution, soil, sand, etc., e.g. potting medium.

**Melting temperature (Abbreviation: T<sub>m</sub>)** - The temperature at which a double-stranded DNA molecule denatures into separate single strands. T<sub>m</sub> is determined by the length of the molecule and its base composition. DNAs rich in G:C base pairs have higher T<sub>m</sub> than A:T rich DNA, because since three hydrogen bonds are formed between G and C, but only two between A and T.

**Micro-array** - A large set of cloned DNA molecules immobilized as a compact and orderly pattern of sub-microlitre spots onto a solid matrix (typically a glass slide). Used to analyse patterns of gene expression, presence of markers, or nucleotide sequence. The major advantage of micro-arrays is the extent to which the process of genotyping can be automated, thereby enabling large numbers of individuals to be simultaneously genotyped at many loci. A similar approach may be used with other immobilized components for other purposes. Synonym: chip or DNA chip.

**Monoclonal antibody (Abbreviation: mAb)** - An antibody, produced by a hybridoma, directed against a single antigenic determinant of an antigen.

**Moribund** - Dying; at the point of death.

**mRNA** - Abbreviation for messenger RNA. The RNA molecule resulting from transcription of a protein-encoding gene, following any splicing (1). The information encoded in the mRNA molecule is translated into a gene product by the ribosomes.



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Necrosis - Pathologic death of one or more cells, or of a portion of tissue or organ, resulting from irreversible damage.

Nosocomial - Denoting a new disorder (not the patient's original condition) associated with being treated in a hospital, such as a hospital-acquired infection.

Nucleoside - A base (purine or pyrimidine) that is covalently linked to a 5-carbon (pentose) sugar. When the sugar is ribose, the nucleoside is a ribonucleoside; when it is 2-deoxyribose, the nucleoside is a deoxyribonucleoside.

Nucleotide - A nucleoside with one or more phosphate groups linked at the 3'- or 5'-hydroxyl of a pentose sugar. When the sugar is ribose, the nucleotide is a ribonucleotide; when it is 2-deoxyribose, the nucleotide is a deoxyribonucleotide. RNA and DNA are polymers of, respectively, ribonucleoside 5'-monophosphates and deoxyribonucleoside 5'-monophosphates. Nucleotides containing the bases adenine, guanine and cytosine (A, G, C) occur in both DNA and RNA; thymine (T) occurs only in DNA, and uracil (U) only in RNA. Ribonucleoside mono-, di-, and triphosphates for which a specific base is not assigned are abbreviated NMP, NDP, and NTP, while deoxyribonucleoside mono-, di-, and tri-phosphates are abbreviated dNMP, dNDP, and dNTP. Otherwise, the "N" is replaced by the base letter abbreviation.

Open reading frame (Abbreviation: ORF) - A sequence of nucleotides in a DNA molecule that has the potential to encode a peptide or protein: comprises a start triplet (ATG), followed by a series of triplets (each of which encodes an amino acid), and ending with a stop codon (TAA, TAG or TGA). The term is generally applied to sequences of DNA fragments, for which no function has yet been determined. The number of ORFs provides an estimate of the number of genes transcribed from the DNA sequence.

ORF - Abbreviation for open reading frame.

Oropharynx - The portion of the pharynx that lies posterior to the mouth; it is continuous above with the nasopharynx via the pharyngeal isthmus and below with the laryngopharynx.

Osteomyelitis - Inflammation of the bone marrow and adjacent bone.

Pandemic - Denoting a disease affecting or attacking the population of an extensive region, country, continent; extensively epidemic.

Papule - A small, circumscribed, solid elevation on the skin.

Passive immunity - Providing temporary protection from disease through the administration of exogenously produced antibody (i.e., transplacental transmission of antibodies to the fetus or the injection of immune globulin for specific preventive purposes).

Pathogen - A disease-causing organism (generally microbial: bacteria, fungi, viruses; but can extend to other organisms: e.g. nematodes etc.). Synonym: infectious agent. See: latent agent.

PCR - see below for polymerase chain reaction.

PCR-RFLP - alternative term for cleaved amplified polymorphic sequence.

Percutaneous - Denoting the passage of substances through unbroken skin, for example, by needle puncture, including introduction of wires and catheters.

Petechia, pl. petechiae - Minute hemorrhagic spots, of pinpoint to pinhead size, in the skin, which are not blanched by pressure.

Petri dish - Flat round glass or plastic dish with a matching lid, used for small-scale culturing of organisms, germinating seeds etc. Also referred to as plates, hence to plate a culture.

PFGE - Abbreviation for pulsed-field gel electrophoresis.

pH - Logarithmic measure of acidity/alkalinity of a solution. A pH of 7 is neutral (e.g. pure water), whereas below 7 is acid and above 7 is alkaline.

Phage - Abbreviation for bacteriophage. See bacteriophage

Phagocytes - Immune system cells that ingest and destroy viruses, bacteria, fungi and other foreign substances or cells.

Phagocytosis - The process by which foreign particles invading the body are engulfed and broken down by phagocytes.

Pharyngeal - Relating to the pharynx.

Pharyngitis - Inflammation of the mucous membrane and underlying parts of the pharynx.

Phenotype - The visible appearance of an individual (with respect to one or more traits) which reflects the reaction of a given genotype with a given environment.

Plaque - A clear spot on an otherwise opaque lawn of bacteria, where cells have been lysed by phage infection.

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**Plasmid** - A circular self-replicating non-chromosomal DNA molecule found in many bacteria, capable of transfer between bacterial cells of the same species, and occasionally of different species. Antibiotic resistance genes are frequently located on plasmids. Plasmids are particularly important as vectors for genetic engineering.

**Point mutation** - A change in DNA sequence at a specific locus. The smallest change involves the substitution, deletion or insertion of a single nucleotide. See: single nucleotide polymorphism.

**Polyacrylamide gel** - Inert electrophoresis matrix, formed by the polymerization of acrylamide monomer in the presence of the cross-linker N,N'-methylene-bis-acrylamide. Gels are usually supported between two glass plates, which need to be removed for post-electrophoresis manipulations. Sometimes referred to incorrectly as acrylamide gels.

**Polyacrylamide gel electrophoresis (Abbreviation: PAGE)** - Ubiquitous method for separating nucleic acids and proteins on the basis of their molecular size. The method relies on the migration through an inert matrix (polyacrylamide gel) of electrically charged molecules as a result of the imposition of an electric field.

**Polyclonal antibody** - A serum sample that contains a mixture of distinct immunoglobulin molecules, each recognizing a different antigenic determinant of a given antigen.

**Polymerase** - An enzyme that catalyses the formation of polymers from monomers. A DNA polymerase synthesizes DNA from deoxynucleoside triphosphates using a complementary DNA strand and a primer. An RNA polymerase synthesizes RNA from ribonucleoside triphosphates and a complementary DNA strand.

**Polymerase chain reaction** - An in vitro method (assay) for enzymatically synthesizing and amplifying defined sequences of DNA in molecular biology. Can be used for improving DNA-based diagnostic procedures for identifying unknown biowarfare agents.

**Polymerase chain reaction (Abbreviation: PCR)** - A widespread molecular biology procedure that allows the production of multiple copies (amplification) of a specific DNA sequence, provided that the base pair sequence of each end of the target is known. It involves multiple cycles of DNA denaturation, primer annealing, and strand extension, and requires a thermostable DNA polymerase, deoxyribonucleotides, and specific oligonucleotides (primers).

**PRNT** - plaque reduction neutralization test is used to quantify the titre of neutralising antibody against a virus. A serum sample is diluted, mixed, briefly incubated with a viral suspension. The suspension is added to a cell culture and covered in a layer of agar or carboxymethyl cellulose to prevent the virus from spreading indiscriminately. Plaque forming units are counted and compared to an untreated virus suspension to determine the neutralizing effect of the serum antibodies. Currently it is considered the "gold standard" for detecting and measuring antibodies that can neutralise specific viruses.

The concentration of serum to reduce the number of plaques by 50% compared to the serum free virus gives the measure of how much antibody is present or how effective it is. This measurement is denoted as the PRNT50 value.

Currently it is considered to be the "gold standard" for detecting and measuring antibodies that can neutralise the viruses that cause many diseases. It has a higher sensitivity than other tests like hemagglutination and many commercial Enzyme immunoassay without compromising specificity.

**Primer** - A short oligonucleotide annealed to a template of single-stranded DNA, providing a doubled stranded structure from which DNA polymerase will synthesize a new DNA strand to produce a duplex molecule.

**Probe** - A labeled DNA or RNA sequence used to detect the presence of a complementary sequence by hybridization with a nucleic acid sample.

**Prokaryote** - A member of the large group of organisms, including bacteria and blue-green algae, in which the chromosome is not enclosed within a nucleus, but instead exists as a linear or circular strand. Prokaryotes do not undergo meiosis and do not have functional organelles such as mitochondria and chloroplasts.

**Pro-phage** - The genome of a bacteriophage integrated into the chromosome of a lysogenic bacterial cell, and replicated along with its host chromosome.

**Protease** - An enzyme that catalyses the hydrolysis of proteins, cleaving the peptide bonds that link amino acids in protein molecules. Synonym: peptidase.

**Protein** - A macromolecule composed of one or more polypeptides, each comprising a chain of amino acids linked by peptide bonds.

**Proteolysis** - Enzymatic degradation of a protein.

**Psychrophile** - A micro-organism that can grow at temperatures below 30 °C and as low as 0 °C. See: mesophile, thermophile.

**Pyrogenic** - Causing fever.

**Radioimmunoassay (Abbreviation: RIA)** - An assay based on the use of a radioactively labeled antibody, where the amount of radiation detected indicates the amount of target substance present in the sample.

**Random amplified polymorphic DNA (Abbreviation: RAPD)** - A PCR-based genotyping technique in which genomic template is amplified with single, short (usually 10-mer) randomly chosen primers. Typical patterns consist of a small number of amplified products of up to 2 kbp in length, which are separated by electrophoresis.

**RAPD** - Abbreviation for random amplified polymorphic DNA.

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**Restriction endonuclease** - A class of enzymes that cut DNA after recognizing a specific sequence. The three types of restriction endonuclease are: I. Where the cut occurs within a random sequence at sites >1kbp from the recognition sequence, and has both restriction and methylation activities. II: Cuts within, or near a short, usually palindromic recognition sequence. A separate enzyme methylates the same recognition sequence. III: Cuts 24-26bp downstream from a short, asymmetrical recognition sequence, requires ATP and has both restriction and methylation activities. Type II enzymes are the class used for most molecular biology applications.

**Restriction enzyme** - Synonym of restriction endonuclease.

**Restriction exonuclease** - A class of enzymes that degrade DNA or RNA, starting from either the 5'- or the 3'-end.

**Restriction fragment** - A shortened DNA molecule generated by the cleavage of a larger molecule by one or more restriction endonucleases.

**Restriction fragment length polymorphism (Abbreviation: RFLP)** - A class of genetic marker based on the detection of variation in the length of restriction fragments generated when DNA is treated with restriction endonucleases. Differences in fragment lengths arise due to genetic variation with respect to the presence or absence of specific recognition site(s). RFLPs were initially detected by Southern hybridization but are now detected by electrophoresis of digested PCR product.

**Restriction map** - The linear arrangement of restriction endonuclease recognition sites along a DNA molecule.

**Restriction site** - Synonym of recognition site.

**Reverse transcriptase** - An enzyme that uses an RNA molecule as a template for the synthesis of a complementary DNA strand. Synonym: RNA-dependent DNA polymerase.

**Reverse transcription** - The synthesis of DNA from a template of RNA, accomplished by reverse transcriptase.

**RFLP** - Abbreviation for restriction fragment length polymorphism.

**RIA** - Abbreviation for radioimmunoassay.

**Ribonuclease (Abbreviation: RNase)** - Any enzyme that catalyses the hydrolysis of RNA.

**Ribonucleic acid (Abbreviation: RNA)** - An organic acid polymer composed of adenosine, guanosine, cytidine and uridine ribonucleotides. The genetic material of some viruses, but more generally is the molecule, derived from DNA by transcription, that carries information (messenger RNA), provides sub-cellular structure (ribosomal RNA), transports amino acids (transfer RNA), or facilitates the biochemical modification of itself or other RNA molecules.

**Ribosomal binding site** - A sequence of nucleotides near the 5' end of a bacterial mRNA molecule that facilitates the binding of the mRNA to the small ribosomal sub-unit. Also called the Shine-Delgarno sequence.

**Ribosomal DNA** - The coding locus for ribosomal RNA. This is generally a large and complex locus, typically composed of a large number of repeat units, separated from one another by the intergenic spacer. A repeat unit comprises a gene copy for each individual ribosomal RNA component, separated from one another by the internal transcribed spacer.

**Ribosomal RNA (Abbreviation: rRNA)** - The RNA molecules that are essential structural and functional components of ribosomes, where protein synthesis occurs. Different classes of rRNA molecule are identified by their sedimentation (S) values. E. coli ribosomes contain one 16S rRNA molecule (1541 nucleotides long) in one (small) ribosomal sub-unit, and a 23S rRNA (2904 nucleotides) and a 5S rRNA (120 nucleotides) in the other (large) sub-unit. These three rRNA molecules are synthesized as part of a large precursor molecule which also contains the sequences of a number of tRNAs. Special processing enzymes cleave this large precursor to generate the functional molecules. Constitutes about 80% of total cellular RNA.

**Ribosome** - The sub-cellular structure that contains both RNA and protein molecules and is the site for the translation of mRNA into protein. Ribosomes comprise large and small sub-units.

**Ribosome-inactivating protein (Abbreviation: RIP)** - A class of plant proteins that inhibit normal ribosome function, and are thus highly toxic. Type 1 RIPs consist of single polypeptide chain proteins; type 2 (e.g. ricin) consist of two proteins linked by a disulphide bridge, one the toxin and the other a lectin that attaches to recognition sites on a target cell.

**RNA polymerase** - A polymerase enzyme that catalyses the synthesis of RNA from a DNA template.

**RVF** -Rift Valley Fever, a viral hemorrhagic fever.

**Saprophytic** - Feeding, absorbing or growing upon decaying organic matter.

**Scarification** -The making of a number of superficial incisions in the skin. It is the technique used to administer tularemia and smallpox vaccines.

**Septic shock** - 1. Shock associated with sepsis, usually associated with abdominal and pelvic infection complicating trauma or operations; 2. Shock associated with septicemia caused by gram-negative bacteria.

**Sequela, pl. sequelae** - A condition following as a consequence of a disease.

## DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

Serum - Blood plasma that has had its clotting factor removed.

Shotgun genome sequencing - A strategy for sequencing a whole genome, in which the genomic DNA is initially fragmented into pieces small enough to be sequenced. Specialized computer software is then used to piece together the individual sequences to create long contiguous tracts of sequenced DNA.

Signal-to-noise ratio - A specifically produced response (signal) compared to the response level (noise) when no specific stimulus (activity) is present.

Single nucleotide polymorphism (SNP) - A genetic marker resulting from variation in sequence at a particular position within a DNA sequence. SNPs are commonly the result of transition changes (A for G, T for C), but also transversions (G or A for T or C) and single base deletions. Such variation is extensive throughout all genomes, and offers the particular advantage of being detectable without the need for gel electrophoresis.

Sodium dodecyl sulphate (Abbreviation: SDS) - A detergent used to solubilize protein and DNA from biological materials. Specific use in sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Solid medium - Nutrient medium solidified by the addition of a gelling agent, commonly agar.

Sonication - Disruption of cells or DNA molecules by high frequency sound waves.

Southern blot - A nitrocellulose or nylon membrane to which DNA fragments previously separated by gel electrophoresis, have been transferred by capillary action.

Southern hybridization - A procedure in which a cloned, labeled segment of DNA is hybridized to DNA restriction fragments on a Southern blot.

Species - A class of individuals capable of interbreeding, but which is reproductively isolated from other such groups having many characteristics in common. A somewhat arbitrary and sometimes blurred classification; but still quite useful in many situations.

Specificity - For diagnostic tests, the ability of a probe to react precisely and uniquely with its target molecule.

Spore - 1. A reproductive cell that develops into an individual without union with other cells; some spores such as meiospores are the product of the germ line, but others are asexual in nature. 2. A small, protected resting body, often synthesized by microorganisms when nutrient levels are low.

Start codon - The codon which specifies the first amino acid of a polypeptide chain and at which the ribosome starts the process of translation. In bacteria, this is either AUG (translated as n-formyl methionine) or, rarely, GUG (valine). In eukaryotes, it is always AUG and is translated as methionine. The start codon sets the reading frame for translation. Synonym: initiation codon.

Stationary phase - The plateau of the growth curve, during which cell number remains relatively constant, following the logarithmic phase. See: growth phases.

Sterile - 1. Medium or object free of viable microorganisms (see: disinfect). 2. Incapable of producing viable gametes.

Stop codon - A set of three nucleotides for which there is no corresponding tRNA molecule to insert an amino acid into the polypeptide chain. Protein synthesis is hence terminated and the completed polypeptide released from the ribosome. Three stop codons are known: UAA (ochre), UAG (amber) and UGA (opal). Synonyms: chain terminator; nonsense codon, termination codon. Strain - A group of individuals derived by descent from a single individual within a species.

Streptavidin - A microbial protein with a high affinity for the B complex vitamin biotin. The specific interaction of these two molecules has been exploited in labeling technology and in applications where a specific molecule needs to be captured or purified.

Stress - Non-optimal conditions for growth. Stresses may be imposed by biotic (pathogens, pests) or abiotic (environment, such as heat, drought etc.) factors.

Stringency - Reaction conditions (notably temperature, salt concentration and pH) that affect the annealing process of single-stranded DNA or RNA to make double-stranded DNA or RNA, or DNA/RNA hybrids. At high stringency, duplexes form only between strands with perfect complementarity; lower stringency allows the annealing of strands with some degree of mismatch.

Structural gene - A gene that encodes a polypeptide, with either enzymatic or structural functions, and that is required for the normal metabolism and growth of a cell or organism.

Sub-culture - Division and transfer of a portion of a culture to fresh medium. Sometimes used to denote the adding of fresh liquid to a suspension culture. Synonym: passage.

Subspecies - Population(s) of organisms sharing certain characteristics that are not present in other populations of the same species.

Substrate - 1. A compound that is altered by an enzyme. 2. Food source for growing cells or microorganisms. 3. Material on which a sedentary organism lives and grows.

Sucrose density gradient centrifugation - A procedure used to fractionate nucleic acids or viruses on the basis of their size.

## DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

**Superantigen** - An antigen that interacts with the T cell receptor in a domain outside of the antigen recognition site. This type of interaction induces the activation of larger numbers of T cells compared to antigens that are presented in the antigen recognition site.

**Supernatant** - The liquid phase remaining after insoluble materials are pelleted by centrifugation or precipitation.

**Synteny** - The occurrence of two or more loci on the same chromosome, without regard to their genetic linkage. Increasingly used to describe the conservation of gene order between related species.

**Tandem repeat** - Two (or more) contiguous identical DNA sequences. The orientation can be either head-to-tail, or head-to-head. Synonyms: tandem array, sequence tandem repeat.

**Thermophile** - An organism which is adapted to high temperatures, such as in hot springs and geysers, smoker vents on the sea floor, and domestic hot water pipes. A wide range of bacteria, fungi and simple plants and animals can grow at temperature up to 50°C; thermophiles grow and reproduce at above 50°C. They can be classified, according to their optimal growth temperature, into simple thermophiles (50-65°C); thermophiles (65-85°C), and extreme thermophiles (>85°C). See: mesophile, psychrophile.

**Thrombocytopenia** - A condition in which there is an abnormally small number of platelets in the circulating blood.

**Tissue culture** - The in vitro culture of cells, tissues or organs in a nutrient medium under sterile conditions.

**Toxoid** - A modified bacterial toxin that has been rendered nontoxic (commonly with formaldehyde) but retains the ability to stimulate the formation of antitoxins (antibodies) and thus producing an active immunity. Examples include Botulinum, tetanus, and diphtheria toxoids.

**Tracheitis** - Inflammation of the lining membrane of the trachea.

**Urticaria** - An eruption of itching wheals, usually of systemic origin; it may be due to a state of hypersensitivity to foods or drugs, foci of infection, physical agents (heat, cold, light, friction), or psychic stimuli.

**Vaccinia** - An infection, primarily local and limited to the site of inoculation, induced in man by inoculation with the vaccinia (cowpox) virus in order to confer resistance to smallpox (variola). On about the third day after vaccination, papules form at the site of inoculation which becomes transformed into umbilicated vesicles and later pustules; they then dry up, and the scab falls off on about the 21st day, leaving a pitted scar; in some cases there are more or less marked constitutional disturbances.

**Variable number tandem repeat (Abbreviation: VNTR)** - A DNA sequence, present as tandem repeats, for which the number of copies varies greatly between unrelated genotypes.

**Varicella** - An acute contagious disease, usually occurring in children, caused by the varicella-zoster virus, a member of the family Herpesviridae, and marked by a sparse eruption of papules, which become vesicles and then pustules, like that of smallpox although less severe and varying in stages, usually with mild constitutional symptoms; incubation period is about 14 to 17 days. Syn: chickenpox

**Viability** - The capability to live and develop normally.

**Viable** - Capable of normal completion of life cycle.

**Viral coat protein** - A protein present in the layer surrounding the nucleic acid core of a virus.

**Virion** - A complete infectious virus particle.

**Virulence** - The degree of ability of an organism to cause disease. The relative infectiousness of a bacterium or virus, or its ability to overcome the resistance of the host metabolism.

**Virus** - An infectious particle composed of a protein capsule and a nucleic acid core (DNA or RNA), which is dependent on a host organism for replication.

**Western blot** - A technique whereby a complex mixture of size-separated proteins is fixed to a solid support, and then probed with a labeled antibody. Useful, for example, for the measurement of levels of production of a specific protein in a particular tissue or at particular developmental stage.

**Zoonosis** - A disease that is communicable from animals to humans.

**Appendix B: Biological Agent Disease Characteristics**

Appendix B: Biological Agent Disease Characteristics						
Disease	Human to human transmission	Infective dose (aerosol)	Incubation period	Duration of illness	Lethality	Persistence of organism
Anthrax	No	8000-1000 spores	1-6 days	3-5 days (fatal if untreated)	High	High
Brucellosis	No	10-100 cells	5-60 days; usually 30-60 days	Weeks to months	Low	High
Glanders	Low	5000-10,000 cells (NHP)	10-14 days	7-10 days (fatal if untreated)	Moderate to high; >50%	High
Melioidosis	Low	50-80 cells (NHP)	1-21 days; up to years	2-3 days (fatal if untreated)	Moderate	High
Plague	Moderate	500-15000 cells	1-7 days; usually 2-3 days	1-6 days (fatal if untreated)	High	High
Tularemia	No	10-50 cells	1-21 days; usually 3-6 days	(fatal if untreated)	Moderate	High
Q fever	Rare	1-10 cells	7-41 days	2-14 days or longer if not treated	Low	High
Smallpox	High	10-100 organisms	7-17 days; average 12 days	4 weeks	High	High
VEE	Low	10-100 organisms	2-6 days	Days to weeks	Low	Low
Viral hemorrhagic fevers	Moderate	1-10 organisms	4-12 days	Death between 7 - 16 days	Moderate - High	Low
Botulism	No	0.003 ug/kg for type A	12 h to 5 days	Death in 24-72 h; lasts for months if not lethal	High	Low (weeks)
SEB	No	0.0004 ug/kg	3-12 h	Hours	Low	Low
Ricin	No	3-5 ug/kg (mouse LD50)	18-24 h	Days	High	High

## Appendix C. Diagnostic Technologies

### Immunoassays.

**Enzyme-linked immunosorbent assay (ELISA):** ELISA involves at least one antibody with specificity for a particular antigen, and often uses two antibodies to sandwich the antigen between a "capture" antibody and a "detector" antibody. The sample (antigen) can be immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody. Between each step, the plate is typically washed to remove any proteins or non-specifically bound antibodies. The assay is typically "developed" by adding an enzymatic substrate that produces a visible signal, which indicates the quantity of antigen in the sample.

1. DIG-ELISA - definition one: Diffusion-in-gel enzyme-linked immunosorbent assay is an adaptation of the standard ELISA procedure where the ability of the antibodies to diffuse from wells in gel in petri dishes and adsorb to an antigen coated to the plastic surface prior to testing. The antigen-antibody reaction is visualized with horseradish-peroxidase conjugated class-specific anti-immunoglobulins.
2. DIG-ELISA - definition two: Digoxigenin-labeled antibody reagents are detected with anti-digoxigenin poly-HRP conjugate. This is the method used for *C. botulinum* toxin testing.

**Time-resolved fluorescence (TRF) assay:** Similar to standard ELISA, time-resolved fluorescence immunoassays use an extension of fluorescence spectroscopy. Here, the fluorescence of an ELISA is monitored as a function of time after excitation by a flash of light. The specific format is the dissociation-enhanced lanthanide fluorescence immunoassay in which long-lasting fluorescent micelles are formed by the dissociation of the complex-bound chelate after adding a low pH enhancement solution. The time resolution can be obtained in a number of ways, depending on the required sensitivity and time resolution. Assays are designed to detect the presence of a compound or biomolecule using lanthanide chelate labeled reagents (typically europium). The fluorescence decay time of these lanthanide chelate labels is much longer than traditional fluorophores, allowing efficient use of temporal resolution for reduction of autofluorescent background. The large Stokes' shift (difference between excitation and emission wavelengths) and the narrow emission peaks contribute to increasing signal-to-noise ratio.

**Lateral flow immunochromatography (LFI/LFA):** Hand-held assays (HHA), "Smart tickets", or lateral flow assays are commercially available and provide potentially useful. The detection technology is based on flow immunochromatography where the test target's antigens that flow over fixed antibodies and are detected with a tagged secondary antibody. The assays are typically designed on nitrocellulose or nylon membranes contained within a plastic or cardboard housing. In the antigen-capture format, a capture antibody is bound to the membrane, and a second labeled antibody is placed on a sample application pad. As the sample migrates down the membrane by capillary action, antigen present in the sample binds to the labeled antibody and is captured as the complex passes the bound antibody. Colloidal gold, carbon, paramagnetic, or colored latex beads are commonly used particles that create a visible line in the capture zone of the assay membrane for a positive result. Turnaround time is usually <5 min. The inherent problem with these assays is the specificity of the antibodies being used and the cross-reactions (false-positives).

**Electrochemiluminescence (ECL):** Similar to standard ELISA, ECL measures a kind of luminescence produced during an electrochemical reaction in a solution. An electrochemically generated intermediate undergoes a reaction that produces an electronically excited state that then emits light upon relaxation to a lower-level state. It generally uses ruthenium complexes, especially [Ru (Bpy)<sub>3</sub>]<sup>2+</sup> (which releases a photon at ~620 nm) regenerating with TPA (tripropylamine) in liquid phase or liquid-solid interface. The use of ECL as a detector for the immunoassay contributes to a generally higher level of sensitivity but the same general immunoassay issue of antibody specificity is present in ECL assays.

**Direct fluorescent antibody (DFA) assays:** DFA is an immunoassay based on a fluorescently tagged antibody. The name derives from the fact that it directly tests the presence of an antigen with the tagged antibody, unlike an indirect fluorescent antibody (IFA) assay or other immunoassays that use an indirect method of detection. The assay requires the use of a fluorescence microscopy to visualize the



bacteria with the bound antibodies. As with all types of fluorescence microscopy, the correct absorption wavelength needs to be determined in order to excite the fluorophore tag attached to the antibody, and detect the fluorescence given off, which indicates which cells are positive for the presence of the virus or bacteria being detected.

**Fluorescence polarization assay (FPA):** The FPA is a simple technique for measuring antigen/antibody interaction and may be performed in a laboratory setting or in the field. It is a homogeneous assay in which analytes are not separated and it is therefore very rapid. The mechanism of the assay is based on random rotation of molecules in solution. Molecular size is the main factor influencing the rate of rotation, which is inversely related. Thus a small molecule rotates faster than a large molecule. If a molecule is labeled with a fluorochrome, the time of rotation through an angle of  $68.5^\circ$  can be determined by measuring polarized light intensity in vertical and horizontal planes. A large molecule emits more light in a single plane (more polarized) than a small molecule rotating faster and emitting more depolarized light. For most FPAs, an antigen of small molecular weight, less than 50 kD, is labeled with a fluorochrome and added to serum or other fluid to be tested for the presence of antibody. If antibody is present, attachment to the labeled antigen will cause its rotational rate to decrease and this decrease can be measured.

**Complement Fixation Test (CFT):** CFT was introduced by Wasserman in 1909 for syphilis serology but later adapted other uses including viral detection. Although CFT is considered relatively simple, it requires specific training because a number of steps are involved. The test consists of two antigen-antibody reactions, one of which is the indicator system. The first reaction, between a known antigen and a specific antibody takes place in the presence of a predetermined amount of complement. The complement is "fixed" by the antigen-antibody complex. The second antigen-antibody reaction consists of reacting sheep red blood cells with hemolysin. When this indicator system is added to the reactants, the sensitized red blood cells will only lyse in the presence of free complement. The CFT assay requires the correct concentrations of hemolytic serum, complement, and antigen, which should be predetermined by titration with previously determined materials.

**Hemagglutination Inhibition Test (HAI or HI):** A wide variety of viruses possess the ability to agglutinate red blood cells of mammalian or avian species. Viruses that can be tested by hemagglutination include influenza, parainfluenza, adenoviruses, rubella, alphaviruses, bunyaviruses, flaviviruses and some strains of picornaviruses. Antibodies against the viral protein responsible for hemagglutination can prevent hemagglutination forming the basis behind the hemagglutination-inhibition test (HAI). The HAI test varies with the different viruses, with some HAI testing being highly specific or less specific (eg. flaviviruses) because of cross-reactions. HAI tests are more sensitive than complement-fixation tests but are less sensitive than some other methods.

**Immunofluorescence (IF):** IF is often used for the rapid diagnosis of virus infections by the detection of virus antigen in clinical specimens, as well as the detection of virus-specific IgG or IgA or IgM antibody. The technique uses fluorescein-labelled antibodies to directly stain the virus, so that the virus infected cells fluoresces under UV illumination. In direct IF, the specimen is stained directly with a labelled antibody against a particular virus antigen. In indirect IF, the specimen is first treated with a non-labelled but specific antibody, followed by a labelled antibody against the first antibody. Indirect IF can also be used to detect induced viral antibodies from infected animals.

### Nucleic Acid Detection Tests:

**Nucleic acid amplification:** The polymerase chain reaction (PCR) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. There have been a number of PCR detection systems that have been developed for the various infectious agents and a large number of method variants are available in the literature. The PCR method most commonly employed for rapid detection currently is based on what is termed real-time PCR. The most common real-time PCR systems uses the typical 2 primers for the amplification of DNA but also a fluorescently labeled 'probe' that anneals between the amplification primers. The probe is hydrolyzed during the amplification reaction releasing the labels, which are then detected by the instrument.

Generally, PCR is based on an enzymatic reaction that involves assembly of nucleosides to fill a space between synthetic oligonucleotides that flank a target nucleic sequence of interest. These

oligonucleotides act as primers for the thermostable Taq polymerase (a polymerase enzyme found in the bacillus *Thermus aquaticus*), that catalyzes the transfer of nucleoside di- or triphosphates into dimer or polymer forms. Repeated cycles (usually 25 to 40) of denaturation of the template DNA (at 94°C), annealing of primers to their complementary sequences (at about 50°C), and extension of nucleosides from the primer (at about 70°C) result in the completion of a nucleotide sequence between the primers. The resulting nucleotide sequence also serves as a target in the second and subsequent rounds of amplification. The repeated process results in the exponential production of the specific target fragment.

There are other versions of real-time PCR that are beyond the discussion presented here.

(1) NOTE: rtPCR has priority in the literature as meaning 'reverse transcriptase' PCR where by RNA is first converted to DNA prior to amplification. Another misnomer is the use of qPCR as a general designator for real-time PCR. qPCR means quantitative PCR which employs the use of a standard curve with real-time PCR to make quantitative estimates of the amount of target DNA present.

(2) Three approaches to real-time PCR are in use:

(a) SYBR Green I (a double-stranded DNA intercalating dye).

(b) 5-exonuclease (enzymatically released fluor).

(c) hybridization probes (fluorescence resonance energy transfer).

While PCR currently has the central role in laboratory nucleic acid amplification assays, there have been a variety of other techniques that may be better suited for different purposes, including better field based diagnostics (3). Assays based on 'isothermal' methodologies such as nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA) and loop-mediated isothermal amplification (LAMP) technologies, are slowly being commercially produced.

**Pulse-field gel electrophoresis (PFGE):** PFGE is a technique used for the separation of large deoxyribonucleic acid (DNA) molecules by applying an electric field that periodically changes direction to a gel matrix. Large DNA molecules do not easily migrate through normal gels under normal electrophoresis conditions; a threshold length exists above 30-50 kb where all large fragments will run at the same rate, and appear in a gel as a single large diffuse band. PFGE improves the migration of large DNA molecules by periodically switching the voltage among three directions; one that runs through the central axis of the gel and two that run at an angle of 120 degrees either side. The pulse times are equal for each direction resulting in a net forward migration of the DNA. This procedure takes longer than normal gel electrophoresis due to the size of the fragments being resolved and the fact that the DNA does not move in a straight line through the gel. Using restriction enzymes to cleave the total DNA into 4 to around 20 pieces and then separating the resulting large fragments of DNA produces band patterns that are based on the total DNA arrangement rather than looking at portions of the DNA that can be visualized with other methods such as ribotyping.

**Multiple-locus variable number tandem repeat analysis (MLVA/VNTR):** MLVA is a method used to perform molecular typing of bacteria. It utilizes the variation in the number of tandem repeated DNA sequences found in many different loci in the genome. A variable number tandem repeat (or VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat (e.g. "AATCCG" repeated a number of times). In MLVA the number of repeats in a set of VNTR loci is assessed. This is achieved by performing PCR of the VNTR loci followed by accurate sizing of the PCR products. The assessed product size is used to calculate the number of repeat units in each locus. The calculated numbers of repeats of the VNTR loci (alleles) are combined into a string which consists of integers e.g. 14-0-2-4-1-7-1-6 and is referred to as the MLVA profile. Each unique MLVA profile is given a MLVA type designation e.g. Ba0021. The MLVA profile can be used for comparison and clustering and often complex assignments can be made. An inherent limitation in the use of MLVA is the erroneous estimates of relationships at larger genetic distances. Some MLVA markers mutate and may cause homoplasy effects, i.e., share of mutational changes for reasons other than common ancestry. This imparts a risk for spurious strain affiliation.

**Amplified fragment length polymorphisms (AFLP):** AFLP-PCR, or just AFLP, is a PCR-based tool for looking at the differences between strains of bacteria. AFLP uses restriction enzymes to digest genomic DNA, followed by ligation of adaptors to the ends of the restriction fragments. PCR of primers complementary to the adaptor sequence to include the restriction site sequence and a few nucleotides inside the restriction site fragments, are then reamplified. The amplified fragments are separated and

visualized on denaturing polyacrylamide gels. The resulting data are scored as presence-absence polymorphisms (2).

**Ribotyping:** Ribotyping involves the electrophoresis of genomic DNA that has been cleaved into "restriction fragments" that contain all or part of the genes coding for the 16S and 23S rRNA. By digesting the genes with a specific restriction enzyme, fragments of different lengths are generated. By performing a gel-electrophoresis with the digested samples, the fragments can be visualized as lines on the gel, where larger fragments are close to the start of the gel, and smaller fragments further down. After electrophoresis, the DNA is transferred to a membrane and annealed with a region of the rRNA operon that has been linked to a detector system, usually and enzyme. This probe reveals the separation of the rRNA genes based on where they were located in the genome relative to the restriction (digestion) sites. The resulting pattern is then compared to other bacterial strains. It is the variations that exist among bacteria in both the position and intensity of rRNA bands that can be used for their classification and identification. These lines form a unique pattern for each species and can be used to identify the origin of the DNA. Ribotyping is a specific application of the general Southern blot technique.

**Repetitive PCR (repPCR):** The Rep-PCR method is based on the use of DNA primers corresponding to naturally occurring interspersed repetitive elements in bacteria, present in multiple copies in the genomes of most gram-negative and several gram-positive bacteria (1). Three families of repetitive sequences have been identified and are most commonly used. The 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element. These sequences appear to be located in distinct, intergenic positions around the genome. The use of these primer(s) leads to the selective amplification of distinct genomic regions located between REP, ERIC or BOX elements. The amplified fragments can be resolved in a gel matrix, yielding a profile referred to as a rep-PCR genomic fingerprint.

**Multi-Locus sequence typing (MLST):** MLST is a nucleotide sequence based approach for the unambiguous characterization of isolates of bacteria and other organisms. MLST characterizes isolates of bacteria using the DNA sequences of housekeeping genes; those genes that are transcribed continually and are required for the maintenance of basic cellular function and are expressed in all cells under normal and patho-physiological conditions. Typically, a 450-500 bp fragments is amplified and sequenced. For each gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST). MLST typically uses 4 to 9 different gene to characterize a strain. Public databases are available on the internet for many different human pathogens.

**"Canonical SNPs" (canSNPs):** A genomic, single nucleotide polymorphisms (SNPs) method where highly stable phylogenetic markers used for identifying long branches or key phylogenetic positions within a characterization scheme. The selection of single, diagnostic canSNPs for specific phylogenetic positions deeply rooted phylogenetic determinations and high resolution discrimination among closely related isolates.

1. **Lupski JR, Weinstock GM.** 1992. Short, interspersed repetitive DNA sequences in prokaryotic genomes. *J Bacteriol* **174**:4525-4529.
2. **Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, et al.** 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* **23**:4407-4414.
3. **Wolcott MJ.** 1992. Advances in nucleic acid-based detection methods. *Clin Microbiol Rev* **5**:370-386.

## Appendix D. Confirmation Levels

1. **Presumptive** identification of a biological threat agent is achieved by the detection of a biological marker using a single test methodology (for example, hand-held assay [HHA]).
2. **Field confirmatory** identification is achieved when two or more independent technologies confirm the identification of a biological agent. This may be an immunoassay (for example, hand held assay [HHA]; electrochemiluminescence [ECL]; enzyme linked immunosorbent assay [ELISA]), a polymerase chain reaction (PCR) result, and/or culture growth/microscopy. A single result from the joint biological agent identification and diagnostic system (JBAIDS) may also be used as a field confirmatory identification. A genomic biomarker must be included.
3. **Theater validation** is achieved using devices, materials, or technologies that detect biomarkers using two or more independent biomarker results (for example, one biomarker is detected by two or more independent methodologies or more than one biomarker is detected by a single methodology). Examples are: (1) hand-held immunological assay plus nucleic acid amplification or (2) nucleic acid amplification using two different biomarkers (for example, gene targets). After a preventive medicine detachment, a combat support hospital (CSH), and/or CBRN reconnaissance assets identifies a biological/clinical specimen as a biological threat agent, the specimen is sent by courier to a specialized laboratory/team with advanced microbiological capabilities and highly skilled medical personnel. These could include laboratories/teams such as an Army Medical Laboratory (AML), United States Air Force (USAF) biological augmentation team, or United States Navy (USN) forward deployable preventive medicine unit (FDPMU) when available in the operational area. These laboratories would conduct initial field confirmatory analysis (quick report) followed by theater validation (more testing and time). If these specialized laboratories/teams are unavailable, biological/clinical specimens that are presumptive positive for a biological threat agent may be forwarded to the nearest reference laboratory. The theater validation laboratory must implement a quality assurance program with independent audits, proficiency testing, data review, document control, demonstration of traceability, electronic sample management, documentation of personnel training, and accreditation (if available).
4. **Definitive** identification is the correlation of a material; chemical, biological, or radiological to a known substance; or, in the case where the substance is previously unknown, the substance is type classified and analyzed. In all cases a definitive identification occurs at a U.S.-based and sanctioned reference laboratory specifically equipped to perform detailed analysis on the type of suspect material to be identified (chemical, biological, or radiological). Definitive identification provides critical information to support decisions regarding national strategic direction and integration. It also supports attribution to implicate or point to the source of the identified material. Definitive identification is performed using the highest level quality assurance measures in a controlled laboratory. Definitive identification or confirmation testing is performed at sanctioned reference laboratories, including reference laboratories of the CDC Laboratory Response Network (LRN). Specific LRN protocols and reagents are proprietary but any definitive identification or confirmation typically follows a general scheme.

## Appendix E. Commercial Identification Systems

### Fatty acid methyl ester (FAME) analysis

The principle of FAME is based upon the analysis of cellular fatty acid compositions, which can be compared with the mean fatty acid composition of the strains used to create the library. After comparison, the identities of unknown microorganisms are determined. The FAME method uses a specific sample preparation procedure and a sophisticated chromatographic system to yield qualitatively and quantitatively reproducible fatty acid composition profiles. This system was developed for microbiologists and it does not require extensive knowledge of gas chromatography. The only commercially available gas chromatography (GC) system dedicated to bacteria identification by fatty acid methyl ester (FAME) analysis is the Sherlock Microbial Identification System (MIS), developed by Microbial ID, Inc. (MIDI). The original database for aerobic bacteria identification was developed by M. Sasser, in 1990.

MIDI has a five-step preparation for standard and rapid extraction methods. Only the harvesting process differs in the standard (40 mg of cell mass) versus the rapid method (20 mg of cell mass). The other steps in the method, saponification (lysis of cells to liberate fatty acids from the cellular lipids), methylation (formation of methylated fatty acids which are necessary for separation and detection), extraction transfer of the fatty acids from the aqueous phase to the organic phase and the base wash (aqueous washes of the organic extraction prior to chromatography analysis) (Sherlock Microbial Identification System Version 6.0 MIS Operating Manual November 2005) were the same. MIDI Instant FAME™ method is a three step process which is methylation, extraction, and separation. The sample process was reduced to less than 3 min by the addition of the reagent for each step. This technique requires only a few cells. Gas chromatography on extracts is performed in approximately 10 min.

The MIDI uses peak naming and pattern recognition algorithms built into the software to identify sample extractions processed on a gas chromatograph. The method uses a retention time/area percentage pairs constructed from chromatographic data. This is compared to an internal table called the Peak Naming Table and each peak is named on relative retention times. Chromatograph problems such as low area, column overload, or poor peak shape, and naming problems can prohibit the library search. Statistical pattern recognition techniques are used to identify the most likely matches (Sherlock Microbial Identification System Version 6.0 MIS Operating Manual November 2005). The retention times for the standard methods, CLIN6 and BTR, versus the rapid methods, RCLIN6 and RBTR, have a decrease amount of retention times that is used for identification.

As mentioned in the *Bacillus anthracis* chapter, MIDI FAME has been approved as an AOAC Official Method for the identification of *B. anthracis* (4).

### Vitek®

The original Vitek was one of the first commercial systems to offer automated antimicrobial susceptibility testing. In 1982, they launched an automated system for *Enterobacteriaceae* that produced results in 8 h. In 1996 they launched the GNI+ card that included 32 biochemical reactions.

The Vitek2 uses test cards that incorporate 64 wells containing individual test substrates. The substrates measure various metabolic activities such as enzyme hydrolysis, acid production, alkalization, and growth in the presence of inhibitory substances and allows identification of more than 2,000 described phenotypes.

The Vitek2 system divides the cards into groups:

1. GN - gram-negative fermenting and non-fermenting bacilli
2. GP - gram-positive cocci and non-spore-forming bacilli
3. YST - yeasts and yeast-like organisms
4. BCL - gram-positive spore-forming bacilli
5. NH - *Neisseria*, *Hemophilus* and other fastidious gram negative bacteria identification
6. ANC - Anaerobic bacteria and coryneform bacteria identification
7. Antibiotic Susceptibility Testing (AST)
  - a. Gram positive antimicrobial susceptibility testing (AST)
  - b. Gram negative antimicrobial susceptibility testing (AST)
  - c. Yeast antimicrobial susceptibility testing (AST)

Operation consists of preparing a suspension of the test organism (typically from a 24 hr culture), loading into the instrument where the card is loaded, incubated, and read periodically until identification is determined (4-18 h).

**Biolog**

The Biolog system uses the principle of carbon substrate use that is detected by redox chemistry, a tetrazolium redox dye, that if the microorganism is capable of utilizing the carbon substrate, the well turns dark indicating reduction of the dye. The fundamental unit in this system is a 96- well plate that has different carbon substrate sources in each well. The system can identify over 2,500 species of aerobic and anaerobic bacteria, yeasts and fungi. The GEN III redox chemistry is applicable to both gram-negative and gram-positive bacteria on one plate; priori knowledge of the gram status of the organism is not needed. The GEN III uses the ability of the cell to metabolize classes of biochemicals and other physiological properties such as pH, salt and lactic acid tolerance, reducing power and chemical sensitivity. While the GN2 system had a Dangerous Pathogens database for identification of dangerous pathogens, the current GENIII does not (1, 2).

**Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer**

Matrix-assisted laser desorption/ionization (MALDI) uses a laser to ionize biomolecules (DNA, proteins, peptides and sugars) and large organic molecules, and the fragments, in the gas phase, are detected by the mass spectrometer after traveling a known distance. The size of the ionized molecules and the time to detection determine a spectra that can be interrelated for identification of molecules, which, in a pattern, can be applied to the identification of bacteria. Sample preparation consists of spotting the sample on a solid target support plate, the sample may be further treated, depending on composition, but is ultimately overlaid with a chemical matrix. Determination of gram status or any other priori knowledge is not needed. Like all automated bacterial characterization systems, their limitation is primarily the composition of the database that supports the interpretation. Diversity in strains, along with the inclusion of organisms outside the normal clinically encountered organisms, will define the specificity, and the ultimate usefulness, of the system (3).

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